IN THE U.S. PATENT AND TRADEMARK OFFICE

Applicants: Brazzell et al. Conf. No.: 9942

Application No.: 10/080,797 Art Unit: 1635

Filed: 21 February 2002 Ex.: J.E. Angell

Title: METHOD FOR TREATING OCULAR NEOVASCULARIZATION

DECLARATION OF YANLIN GUO

I, Yanlin Guo, do hereby declare and state:

1) I am on faculty in the Department of Biological Sciences at the University of Southern Mississippi.

2) I have been studying, cancer cell biology, vascular biology and angiogenesis since 1998. I have been investigating the mechanisms of angiogenesis and methods of treating aberrant vessel development.

3) My qualifications are set forth in my curriculum vitae.

4) I am familiar with endostatin. I also am aware of the subject matter of the this patent application. I was in communication with one of the co-inventors and was quite surprised when he reported the successful use of endostatin in the eye. Specifically, the prevailing thought at the time was that the anti-cancer activity of endostatin was extremely controversial.

5) The initial reports describing the biology of endostatin were generated by the Harvard laboratory of Dr. Judah Folkman. Based on reports from this group that

endostatin was effective in treating cancer in mice, the first clinical indication was in the field of oncology.

6) However, the initial observations from the Folkman laboratory could not be repeatedly confirmed by other laboratories, and a growing body of negative data led to increasing skepticism regarding the potential use of endostatin to treat cancer. These concerns were portrayed in a 1998 news clipping from the Wall Street Journal (Exhibit 1).

7) Nevertheless, Entremed acquired rights to commercialize endostatin. Entremed was developing endostatin as a cancer therapeutic and initiated clinical trials. The clinical results were disappointing (Exhibit 2), and Entremed ultimately out-licensed the rights to endostatin (Exhibit 3).

8) Independently, several papers reported unsuccessful attempts to confirm the suggestion that endostatin has a therapeutic anti-cancer effect.

9) For example, in 2001, (Jouanneau et al., J Neuro-Oncol 51, 11, 2001) (Exhibit 4), investigators from CNRS and INSERM in France, and MIT and Harvard in the US reported that recombinant, biologically-active endostatin protein injected subcutaneously daily for 12 days was ineffective in killing a human neuroblastoma cell line in a mouse model. Recombinant endostatin was detectable in the serum of the treated mice. At best, there was retardation of tumor growth; however, the results were not statistically significant and tumor death was not observed. Instead, the tumors continued to grow over time. Please see Figure 3 of Jouanneau et al.

10) In 2002, two well-controlled studies, described below, generated several commentaries. In a commentary in Science (Science, 295, 2198, 2002) (Exhibit 5), Marshall noted that the studies, which were conducted, in part, at Harvard and at Genetix Pharmaceuticals, a biotech company interested in commercializing a therapeutic use of endostatin, revealed that endostatin had no anti-angiogenic or anti-cancer effect in vivo.

In a commentary in BioMedNet News (Exhibit 6), Apoorva Mandavilli noted that an NCI investigator, Melinda Hollingshead, was one of several scientists unable to replicate the observations of the Folkman lab. Dr. Hollingshead stated that endostatin will not be accepted as a mainstream cancer therapy until studies have clarified its mechanisms and resolved the inconsistencies between Folkman's lab and other labs. She said that the scientific controversy over endostatin has not even begun to be resolved.

11) The two papers reporting no biological activity for endostatin in vivo, referred to above, appeared in the journal Molecular Therapy. The editors of Molecular Therapy commented that it was not common practice to publish negative data (Exhibit 7). However, the editors concluded that the publication of negative data is mandated when the studies are scientifically sound and have an immediate impact on medical research. Thus, it was important to publish the two manuscripts that detail the negative endostatin data as a purported cancer treatment. The two articles are summarized below.

12) In Pawliuk et al. (Molecular Therapy 5, 345, 2002) (Exhibit 8), scientists from Harvard, Genetix and The Karolinska Institute engineered hematopoietic stem cells to express endostatin, which was shown to be biologically active via an in vitro assay. Following administration of the stem cells to mice, circulating recombinant endostatin was detectable in the serum by ELISA. However, despite the high serum levels of endostatin, no inhibition of angiogenesis or anti-tumor activity was observed. The investigators also used a corneal micropocket assay to determine if endostatin expression in the mice had any anti-angiogenic effect in vivo. A pellet containing FGF-2 was implanted into the cornea to induce neovascularization. The investigators found no difference in neovascularization between the mice expressing endostatin and the control mice. In addition, no difference in wound healing was observed between the experimental animals and controls. Thus, in this in vivo gene therapy setting, endostatin showed no anti-tumor activity or anti-angiogenic activity.

13) In Eisterer et al. (Molecular Therapy 5, 352, 2002) (Exhibit 9), investigators from the British Columbia Cancer Agency, Genetix, MIT and Harvard

reported expression of endostatin by transduced cells in SCID mice, but there was no evidence that endostatin was effective in halting the growth of engrafted human lymphocytic leukemia cells obtained from four different patients.

14) Then Bachelot et al. (Curr Med Chem-Imun Endo & Metab Agents 2, 233, 2002) (Exhibit 10) summarized the preclinical development of endostatin as an anticancer agent. Several studies by independent teams did not show consistent tumor regression in a mouse model. More recent studies (relative to the 2002 publication of Bachelot et al.) failed to show any significant antitumor activity. Some explanations for the lack of confirmation of the Folkman results include: 1) the need to produce the endostatin in E. coli since that was the production method used by the Folkman lab; 2) the differences in angiogenesis among tissues and the dynamic changes that occur during the different phases of blood vessel growth; and 3) the notion that detection assays may not accurately reflect the levels of biologically active endostatin. The review concludes by stating that there are a great deal of unknowns relating to endostatin and any anti-tumor activity that the molecule might have. Many unanswered questions remain and any therapeutic use of endostatin to treat cancer in humans is impossible at that time.

15) Thus, as early as the Wall Street Journal article in 1998 and the Jouanneau article in 2001, there was growing suspicion concerning the anti-angiogenic potential of endostatin for treating cancer.

16) Based on my research and understanding of this subject, angiogenesis is an extremely complex process that may vary from tissue to tissue. The combination effect of pro-angiogenic and anti-angiogenic factors may vary under different physiological or pathological conditions. Hence, the knowledge of angiogenesis derived from one tissue does not necessarily apply to an understanding of angiogenesis in another tissue.

17) Therefore, any reports of angiogenesis or anti-angiogenesis in one tissue, such as cancer, may not necessarily be applicable to another tissue, such as the eye. So,

even if the observations of the Folkman laboratory of an anti-cancer effect in mice could be proven, that would not suggest that endostatin will have any anti-angiogenic effect in a normal, nonmalignant tissue, such as the eye, without thorough investigation.

18) To summarize, the scientific evidence support the conclusion that as early as 1998, there was substantial skepticism concerning the use of endostatin for the treatment of cancer, and subsequent studies failed to demonstrate efficacy for endostatin in the treatment of cancer in humans (Exhibit 2). At best, there might have been a suggestion of an anti-tumor activity in mice; however, that observation was not reproducible in many laboratories. The unsuccessful cancer studies, in connection with what was known about the heterogeneous nature of angiogenesis, support the further conclusion that, at that time, there was no reproducible evidence to suggest that endostatin would have any therapeutic activity in non-cancerous tissues, such as the eye.

19) Whereas it is possible that studies in the future may demonstrate a role for endostatin in the treatment of human cancer, it is clear that in the late 1990s there was no sufficient reason to expect in vivo efficacy with endostatin, particularly in an ocular setting. Thus, it is quite remarkable that the inventors found such potent and reproducible efficacy with endostatin for ocular neovascularization.

All statements made herein are based on my own knowledge and understanding of the subject, and that all statements made on information and belief are true; and further that the statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing therefrom.

3/26/07
Date

Yanlin Guo, Ph.D.

Curriculum Vitae

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EDUCATION

1978-1982 **B.S.**, Biological Sciences, Hebei Normal University, China.
1987-1988 **Diploma,** Advanced Intensive English Program, Sichuan Foreign Language Institute, China.
1991-1996 **Ph. D.,** Molecular Biology & Biochemistry, Botany Department, The University of Texas at Austin.
1996-1999 **Postdoctoral Fellow,** Department of Biochemistry & Biophysics School of Medicine, University of Pennsylvania.

POSITIONS AND EMPLOYMNET

1982-1988 **Research/Teaching/Assistant**, Biology Department, Hebei Normal University, China.
1988-1990 **Visiting Research Scientist**, Botany Department, The University of Texas at Austin.
1990-1991 **Research Associate/Lecturer,** Biology Department, Hebei Normal University, China.
1991-1996 **Research/Teaching Assistant**, Botany Department, The University of Texas at Austin.
1996-1999 **Postdoctoral Fellow**, Department of Biochemistry & Biophysics, University of Pennsylvania School of Medicine.
1999-2004 **Research Assistant Professor,** Sol Sherry Thrombosis Research Center, Temple University School of Medicine.
2004-present **Assistant Professor,** Department of Biological Sciences, The University of Southern Mississippi

HONORS AND AWARDS

1988-1989 **Scholarship for Study Abroad,** Hebei Normal University, China
1995 **Harold E. Bold Award, Excellence in Teaching,** Botany Department, The University of Texas at Austin
1995 **Memorial Fellowship, Excellence in Research,** Botany Department, The University of Texas at Austin.
1995-1996 **Continuing Fellowship,** Graduate School, The University of Texas at Austin.
1997-1999 **NIH Training Grant,** School of Medicine, University of Pennsylvania.
1999 **ASBMB Travel Award,** The American Society for Biochemistry and Molecular Biology.

2005 **Summer Faculty Research Grant**, The University of Southern Mississippi.
2007-2008 **A.K. Lucas and E.G. Lucas Endowment for Faculty Excellence Award,** The University of Southern Mississippi.

MEMBERSHIP IN PROFESSIONAL SOCIETIES

The American Heart Association, Vascular Biology Council Member
International Society on Thrombosis and Haemostasis
International Society on Stem Cell Research
American Physiological Society

PUBLICATIONS

A. Original Refereed Articles

Guo, Y-L, Roux, SJ, Partial purification and characterization of a Ca^{2+}-dependent protein kinase from the green alga, *Dunaliella salina*. ***Plant Physiol*** 1990, 94:143-50

Guo, Y-L, Roux, SJ, Further characterization of the calcium-dependent protein kinase from the green alga, *Dunaliella salina*. ***Acta Phytophysiologia Sinica*** 1992, 18:300-08

Guo, Y-L, Roux, SJ, Partial purification and characterization of an enzyme from pea nuclei with protein tyrosine phosphatase activity. ***Plant Physiol*** 1995, 107:167-75

Guo, Y-L, Roux, SJ, Partial purification and characterization of a type 1 protein phosphatase in purified nuclei of pea plumules. ***Biochemical J*** 1996, 319:985-91

Guo, Y-L, Peng, M, Kang, B, Williamson, JR, Inhibition of thrombin-stimulated cell proliferation by ceramide is not through inhibition of extracellular signal-regulated protein kinase. ***Biochem Biophys Res Commun*** 1997, 240: 405-08

Guo, Y-L, Terry, ME, Roux, SJ, Characterization of a cytosolic phosphatase from pea plumules having significant protein tyrosine phosphatase activity. ***Plant Physiol Biochem*** 1998, 36:269-78

Guo, Y-L, Baysal, K, Kang, B, Yang, L-J, Williamson, JR, Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis induced by tumor necrosis factor-α in rat mesangial cells. ***J Biol Chem*** 1998, 273:4027-34

Guo, Y-L, Kang, B, Williamson, JR, Inhibition of the expression of mitogen-activated protein phosphatase-1 potentiates apoptosis induced by tumor necrosis factor-α in rat mesangial cells. ***J Biol Chem*** 1998, 273: 10362-66

Yang, LJ, **Guo, Y-L**, Li, Q-Y, Maloney, AJ, Steinhauer, M, Williamson, JR, Epidermal growth factor and angiotensin II signaling to extracellular signal-regulated protein kinase in rat liver epithelial WB cells. ***Biochem Pharmcol*** 1999, 57: 425-32

Guo, Y-L, Kang, B, Yang, L-J, Williamson, JR, Tumor necrosis factor-α and ceramide induce cell death through different mechanisms in rat mesangial cells. ***Am J Physiol*** 1999, 276: F390-F97

Guo, Y-L, Kang, B,Williamson, JR, Resistance to tumor necrosis factor-α cytotoxicity can be achieved through different signaling pathways in rat mesangial cells. ***Am J Physiol*** 1999; 276: C435 -C41

Guo, Y-L, K., Kang, B., Han, J., Williamson, JR, p38 MAP kinase protects rat mesangial cells from TNF-α-induced apoptosis. ***J Cell Biochem*** 2001, 82: 556-565.

Guo, Y-L, Wang, S, Colman, RW, Kininostatin, an angiogenic inhibitor, inhibits proliferation

and induces apoptosis of endothelial cells. ***Arterioscler Thromb Vasc Biol*** 2001, 21: 1427-1433.

Wang, S, Hasham, MG, isordia-salas, I, Tsygankov, AY, Colman, AR and **Guo, Y-L,** Up-regulation of cdc2 and cyclin a during apoptosis of endothelial cells induced by cleaved high molecular weight kininogen. ***Am J Physiol*** 2003, 284: H1917-H1923

Guo, Y-L, Wang, S, Colman, RW, Apoptotic effect of the cleaved high molecular weight kininogen is regulated by extracellular matrix proteins. Submitted in revised version to ***J Cell Biochem*** 2003, 89:662-632

Yang, BH, Cao, DJ, and Colman, RW. **Guo, Y-L**. Different roles of ERK and p38 MAP kinases during tube formation from endothelial cells in 3-dimensional cell culture. ***J Cell Physiol.*** 2004, 200:360-369

Cao, D.J. **Guo, Y-L,** Colman, RW. Urokinase-type plasminogen activator receptor is involved in mediating the apoptotic effect of cleaved high molecular weight kininogen. ***Circulation Res*** 2004, 94:127-1234.

Song, JS, Sainz,IM, Cosenza,SC, Isordia-Salas, I, Bior, A, Bradford, HN, **Guo, Y-L**, Pixley, RA, Reddy, EP, Colman, RW. Inhibition of tumor angiogenesis in vivo by monoclonal antibody targeted to domain 5 of high molecular weight kininogen. ***Blood.*** 2004, 104: 2065-2072

Jiang, X, **Guo, YL,** and Bromberg. Formation of Tissue Factor-Factor VIIa-Factor Xa Complex Prevents Apoptosis in Human Breast Cancer Cells. ***Thrombosis and Haemostasis,*** 2006, 96:196-201

Guo, Y-L and Yang, B. Altered cell adhesion and cell viability in mouse embryonic stem cells deficiency of p38a MAP kinase. ***Stem Cells Dev.*** 2006, 15: 655-664

Yang, B. and **Guo,Y-L**, p38α and p38β MAP Kinases Cooperatively Regulate Intracellular Vacuole Formation in Endothelial Cells. Submitted, 2007

Cao, D.J. **Guo, Y-L,** Colman, RW. Cleaved high molecular weight kininogen inhibits human endothelial cells capillary lumen structure formation by inhibiting ERK activation and cell migration. Submitted, 2007.

B. Review Articles (Selected)

Roux, SJ, **Guo, Y-L**, & Li, H, Characterization of two calcium-dependent protein kinases implicated in stimulus-response coupling in plants. In: ***Cur Top Plant Biochem Physiol*** (Ed) D. Randall & D. Blevins 1990; 9:129-40

Guo, Y-L, Sun, D-Y, Recent progress in the study of intracellular signaling systems. ***Prog Biochem Biophys*** 1995; 22: 217-23

Guo, Y-L, Wang, S, Colman, RW, Kininostatin as an antiangiogenic inhibitor: what we know and what we don't know. ***Int. Immuno Pharmocol*** 2002; 1931-40

Guo, Y-L and Colman, RW. Two faces of high molecular weight kininogen (HK) in angiogenesis:bradykinin turns it on and cleaved HK (HKa) turns it off. ***J Thromb Haemost*** 2005, 3:670-676.

C. Book Chapters

Cell signaling Systems. (1st Ed). Edited by Sun, D-Y and **Guo, Y-L**. 1993, Beijing, Science Press

Cell signaling Systems. (2nd Ed). Edited by Sun, D-Y, **Guo, Y-L** and Ma, L-G. 1998, Beijing, Science Press

Cell signaling Systems. (3rd Ed). Edited by Sun, D-Y, **Guo, Y-L** and Ma, L-G. 2001, Beijing, Science Press

Nucleic Acids As Regulatory Molecules. Haung, H., Zhang, C.J. and **Guo, Y-L.** In ***RNA Interference: Application to Drug Discovery and Challenges to Pharmaceutical Development.*** Edited by Teresa N. Faria, Siew Peng Ho, and Oliver Steinbach. 2007, John Wiley & Son Inc.

E. Abstracts (Selected)

Guo, Y-L., Williamson, J.R. (1998) Correlation between sustained c-Jun N-terminal protein kinase activation and apoptosis-induced by tumor necrosis factor-alpha in rat mesangial cells.***Specificity in Signal Transduction, Keystone Symposium*** p.54.

Guo, Y-L., Kang, B., Williamson, J.R. (1999) p38β MAP kinase protects rat mesangial cells from TNF-α induced apoptosis. ***Annual Meeting of the American Society for Biochemistry and Molecular Biology.***

Guo, Y-L, Wang, S, Colman, R.W. (2001) Kininostatin, an angiogenic inhibitor, inhibits proliferation and induces apoptosis of endothelial cells. ***XVIII Congress of the International Society on Thrombosis and Haemostasis.***

Wang, S, Colman, R.W. **Guo, Y-L,** (2001) Apoptotic effect of the cleaved high molecular weight kininogen is associated with its antiadhesive activity. ***Blood***, 98: 30a

Guo, Y-L, Wang, S, Colman, R.W. (2001) Up-regulation of cdc2 kinase and cyclin a during apoptosis of endothelial cells induced by angiogenic inhibitor kininostatin. ***Blood,*** 98: 32a

Guo, Y-L, Yang, B (2006). Altered cell adhesion and cell viability in mouse embryonic stem cells deficiency of p38a MAP kinase. *FASEB J.A1459*

RESEARCH SUPPORT

A. Ongoing Research Support

NIH/NHLBI R15 HL081126-01 (Guo, PI) 8/1/05 – 7/31/08

"p38α and p38β MAP Kinases in Endothelial Cells"

This project investigates the specific roles of p38 α and β MAP kinases in the regulation of primary endothelial cell activities related to angiogenesis.

NIH/NHLBI R21 HL082731-01A1 (Guo, PI) 7/1/06 – 5/31/08

"p38α MAP Kinase in Mouse Embryonic Stem Cells" This project investigate the role of p38α in the regulation of mouse embryonic stem cell differentiation.

B. Pending Research Support (pending for final budget approval)

NIH/ NCI R21 (Huang, PI, Guo, Co-PI)
"Folate Receptor-Mediated siRNA Delivery to Cancer Cells"

C. Completed Research Support

The American Heart Association 0265404U (Guo, PI) 7/1/02- 6/30/04

"Signaling mechanisms mediating the apoptotic effect of cleaved high molecular weight kininogen" The major goal of this study is to investigate the signaling pathways that mediate the antiangiogenic effect of cleaved high molecular weight kininogen (HKa) in endothelial cells.

Pennsylvania Department of Health (Guo, PI) 1/1/03-6/30/04

Program Project: Regulation of Tumor Angiogenesis by Kininostatin. Program PI, R. Colman
Project Core A: (Guo, PI). "Three-dimensional (3D) cell culture as an in vitro angiogenesis model"

NIH/NCI R01 CA83121 (Colman, PI; Guo, Co-investigator) 7/11999-6/30/2004

"Kininogen-uroikinase Receptor Interaction in Tumor Angiogenesis"
This grant explores the anti-angiogenic effect of peptides from kininogen on the growth of tumors in animal models.

The University of Southern Mississippi, Dean's Research Initiative 2004

The University of Southern Mississippi, Faculty Summer Research Grant 2005
"Characterization of p38 alpha-knockout Mouse Embryonic Stem Cells"

The University of Southern Mississippi, (MFGN) 2005
Summer research grant for undergraduate students (REO program)

The University of Southern Mississippi Startup fund 9/01/04-8/30/06

"Signaling mechanisms that regulate angiogenesis"

REVIEW FOR GRANTING AGENCIES, JOURNALS, AND PUBLISHERS

A. Granting Agencies

Health Research Board of Ireland
Biotechnology and Biological Sciences Research Council of United Kingdom.

B. Scientifc Journals

Cancer Resaerch, Clinical Cancer Research, American Journal of Physiology, Molecular Cancer Therapeutics, FEBS letters, Stem Cells, Free Radical Biology & Medicine.

C. Review for Publishers

Introductory Cell and Molecular Biology Textbook (Kreuzer & Karp). John Wiley & Sons, Inc.

INVITED LECTURES

Temple University School of Medicine. Thrombosis Research Center. Signaling Mechanism of the Angiogenic Inhibitor, Cleaved High Molecular Weight Kininogen (2003)

The University of Southern Mississippi, Department of Biological Sciences. ERK and p38 MAP Kinases in the Regulation of Angiogenesis (2004)

The University of Southern Mississippi, MRSEC group. Three-dimensional cell culture system as an angiogenesis model (2005).

The University of Southern Mississippi, MFGN group.Regulation of Angiogenesis by p38 MAP Kinases (2005).

The University of Southern Mississippi, Department of Biological Sciences. Embryonic Stem Cells: What We Know and What We Don't Know (2005).

Mississippi State University, Dept. of Biochemistry and Molecular Biology. Regulation of Angiogenesis by p38 MAP Kinases (2006).

Hebei Normal University, China. College of Life Sciences. The role of p38 MAP kinase in the regulation of embryonic stem cells (2006).

NATIONAL AND INTERNATIONAL MEETING PRESENTATIONS (selected)

Keystone Symposium, Specificity in signal transduction. Mar. 1-7, 1998, Lake, Tahoe, NV, USA

Annual Meeting of the American Society for Biochemistry and Molecular Biology. Mar 1999, San Francisco, CA, USA

XVIII Congress of The International Society on Thrombosis and Haemostasis. July 6-12, 2001, Paris, France

29th Annual Philadelphia Workshop on Hemostasis, Thrombosis and Atherosclerosis. Nov. 16, 2001, Philadelphia, PA, USA

43rd Annual Meeting of the American Society of Hematology. Dec. 7-12, 2001, Orlando, FL, USA

16th International Conference on the Kallikrein-Kinin System. May 26-31, 2002 Charleston, SC, USA

XIX Congress of The International Society on Thrombosis and Haemostasis. July 12-18, 2003, Birmingham, United Kingdom

Experimental Biology 2004. April 17-21, 2004, Washington DC, USA

Experimental Biology 2006. April 1-5, 2006, San Francisco, USA

Novel Cancer Approach Stumbles As Others Fail to Repeat Successes

By Ralph T. King, Jr., Staff reporter of the Wall Street Journal

November 12, 1998

BOSTON -- At a cancer conference hosted by Michael Milken in September, noted researcher Judah Folkman treated scientists to a mesmerizing presentation of his latest work, with vivid lab photos and graphs showing antitumor agents to be unambiguously effective in mice. Crowding around afterward were Mr. Milken, the former junk-bond king, as well as Intel Corp. Chairman Andrew Grove, a CNN cameraman and an attendee who clapped Dr. Folkman on the back and said, "Beautiful talk, Judah."

Dr. Folkman is used to warm receptions. As the father of the concept of attacking tumors by cutting off their blood supply, he has become one of the world's most visible cancer fighters. At Children's Hospital here, people stand aside as he passes in hallways hung with his citations. A 1997 Fortune profile pegged him as a likely Nobel Prize winner. And last May he and his cancer strategy burst into the national consciousness after being prominently featured in a New York Times story. In an instant, hopes of countless cancer patients brightened, and Dr. Folkman was being treated as a medical prophet.

But the public acclaim stands in contrast to doubts and frustration within parts of the research community. A number of experts say they haven't been able to verify Dr. Folkman's findings: that an agent called endostatin can cause large tumors in mice to shrink and lie dormant, and that this plus a second agent called angiostatin can make such tumors vanish. In science, the critical test of experimental data is that they can be reproduced by other scientists working independently.

One collaborator, the National Cancer Institute, is so concerned about its inability to do so that it had begun to form a panel of outside experts to investigate, when Dr. Folkman agreed to give institute scientists a 10-day demonstration in his lab later this month. Dr. Folkman also was queried about the issue by Boston's Dana-Farber Cancer Institute following Internet rumors he would retract his research in the scientific journal Nature -- rumors that Dr. Folkman says have no basis. (Nature declines to comment.) Meanwhile, Genentech Inc. scientists tried to duplicate Dr. Folkman's results for a year, then gave up. Says Genentech Chief Executive Arthur Levinson: "We want to know what's going on."

EntreMed Inc., a tiny biotech company that licensed the two biological agents, hired Harvard cell biologist Bjorn Olsen to look into endostatin. Using endostatin variants he made, he couldn't reproduce Dr. Folkman's results, Dr. Olsen says. Dr. Olsen, who had

originally helped the Folkman lab isolate the substance, suggests it would be foolish to test the agent in humans without a better understanding of how it works and possible modification.

Human tests don't appear likely soon. The National Cancer Institute has delayed human testing of endostatin for at least a year. As for angiostatin, Bristol-Myers Squibb Co., which licensed it from EntreMed in 1995, has no set timetable for human tests because "it requires a lot more work," says William Koster, senior vice president for drug discovery.

For more than two years, Bristol-Myers couldn't produce consistent angiostatin results, with one batch showing some effect but the next showing little or nothing. Recently, the company did succeed in making small batches that reliably slow tumor growth in mice, Dr. Koster says, but he adds that practical problems in trying the substance on human beings abound. Says the company's senior vice president of pharmaceutical development, Christopher Cimarusti: "There's a difference between a lab curiosity and something you can take forward in man."

The Caveats

That distinction, while eminently clear to scientists such as Dr. Folkman, is often lost in the public's understanding of the immensely complicated quest for a cancer cure. Dr. Folkman didn't seek the publicity he received for his efforts in May, and he declined numerous requests for television interviews afterward. He agonizes over false hopes raised in cancer patients, and he makes a point of stressing that it is a long journey from lab to bedside for any drug candidate, including his own.

Yet some researchers who have worked or competed with Dr. Folkman say he has been so single-minded in promoting his theories that he has promised too much too soon. Dr. Folkman has been publicizing antitumor findings and securing large corporate grants since 1972, but no commercial cancer drugs have resulted.

When one compound hasn't lived up to its hopes, he has proposed a new and better one. His career thus seems to illustrate an inherent tension within modern-day, high-stakes medical science: the conflict between the advisability of great caution in reporting data and the need to sell projects to sponsors so the promising research can continue.

"Judah's really opened up the field, but some scientists think he's the boy who cried wolf," says David Cheresh, a Scripps Research Institute scientist also noted in the cancer field.

Dr. Folkman says his results have been reproduced again and again in his own laboratory. At least three different groups in his lab have done so using scientific protocols he has perfected over 30 years. If others have difficulty reproducing his experiments, he says, this is simply because of the extraordinary skill levels required in producing the complex molecules and working with them. There are many techniques that must be learned over

time, he says. Likening such skills to those that produced a Stradivarius violin, he notes, "There's no way you can get that skill in a short time."

As for promising too much, he says, "In the pharmaceutical world, one in 1,000 drugs makes it all the way. Does that mean it's crying wolf? If it fails, it fails for unpredictable reasons, so I don't see what the complaint is."

A Surgeon First

Before turning to cancer research, Dr. Folkman distinguished himself as a wunderkind surgeon, becoming chief of surgery at Children's Hospital at age 34. Seeing hundreds of bloody tumors, he came up with the idea of starving them of blood-blocking "angiogenesis," the process by which new blood vessels spring up to feed a growing tumor. He published dozens of provocative papers, trained many of the field's prominent investigators, and won more than 50 scientific awards. He began to teach at Harvard and built his lab into one of Harvard's biggest, with a budget of $8 million a year and more than 100 scientists.

Now 65, Dr. Folkman still works 75-hour weeks and makes time after hours to return calls from cancer patients. He has never tried to get rich on his research, consigning all rights and license payments to Harvard-affiliated Children's Hospital in accordance with Harvard policy. "Judah, with his vision, has made a tremendous contribution," says Isaiah Fidler, a department chairman at the University of Texas' M.D. Anderson Cancer Center in Houston. "He is one of the most decent, compassionate people I have ever met in my life, and he's a brilliant scientist."

But right from the start, Dr. Folkman had to battle for respect -- and funding. His approach was unconventional, and big government grants weren't forthcoming. In response, he vigorously promoted his findings to drug companies. In 1972, he used a time-lapse film to show medical writers how blocking something called tumor angiogenesis factor stunted a tumor. Monsanto Co. gave Dr. Folkman and a colleague $23 million, a grant so large that Harvard formed a panel to monitor it.

The company today describes the project as one "that taught Monsanto a lot about how it can work with institutions," but not one that led to products. Dr. Folkman says, "They quit too early; they could have had the whole field." The collaboration ended in 1984.

Other Compounds

Dr. Folkman had by then moved on to a new crop of antitumor agents. A Boston-area biotech company, Repligen Corp., financed $40 million of Folkman-led research on one of them, called "platelet factor 4," only to find that it wasn't effective in humans, at least at the doses tested. Dr. Folkman says, "It was a very good drug, but you can't get it potent enough."

Dr. Folkman stirred brief excitement with two well-known substances, cortisone and the blood thinner heparin. His 1983 Science magazine report on the combination told of "complete regression" of tumors in most mice tested. But in 1985, two teams of researchers reported they hadn't been able to reproduce his results.

To Dr. Folkman, this just illustrates his point that it can take time for others to learn how. He says that while the two labs indeed couldn't match his results, numerous other labs from several countries eventually did. But the combination proved too toxic, and went no further.

At any rate, by this time Dr. Folkman had pulled his next experimental compound literally out of thin air. It was an airborne fungus that landed in a petri dish set up for testing in his lab. That serendipity, reminiscent of the discovery of penicillin, launched a $1 million-a-year deal with Takeda Pharmaceuticals of Japan. But human trials of the fungus have continued inconclusively for almost seven years, while the National Cancer Institute gave up testing the fungus in cancer patients after it left some too dizzy to walk, says an investigator. Dr. Folkman believes the fungus may prove useful at lower dosages in conjunction with other therapies.

Looking for Support

Eventually, Dr. Folkman's lack of commercial success in cancer began to catch up to him. At one point in late 1992, he says, he had to temporarily divert certain grant money to pay researchers' salaries.

The quest for funds was a time-consuming burden. Drug-company envoys trooped through the lab and his co-workers were asked to give demonstrations lasting hours, says a former cell biologist in his lab. After striking out with big companies, Dr. Folkman says, he finally mailed out fliers to small companies.

One, EntreMed, of Rockville, Md., got aboard, but it was too small to develop a drug by itself. Then in early 1995, Bristol-Myers Squibb gave Dr. Folkman its annual cancer-research award. At the award dinner, Dr. Folkman pitched his lab's newly discovered angiostatin to the company's research chief. Bristol-Myers licensed the agent but didn't pursue it nearly as aggressively as Dr. Folkman wanted. Last year when the Folkman lab announced a second substance that could cut off tumor's blood supply -- endostatin -- EntreMed began studying it, as did the National Cancer Institute, Genentech and others.

There things stood when Dr. Folkman's efforts received a burst of publicity in May. EntreMed stock briefly soared sevenfold. Top brass at Bristol-Myers, caught by surprise, contacted their own scientists, who called in collaborators from Dr. Folkman's lab, EntreMed and the National Cancer Institute. "We decided to work very hard to bring angiostatin to a successful conclusion, to really understand it and decide what role it had," says Bristol-Myers' Dr. Cimarusti.

Complex Molecules

Just creating the complex substance is a laboratory challenge, he says. "Angiostatin is a very, very large molecule. It's comparable to the hardest we have ever attempted here." Scientists must join a string of protein constituents so they fold into a precise three-dimensional shape.

Endostatin is even trickier to work with. Dr. Folkman says it is so fragile that to get it from his Boston lab to the National Cancer Institute in Bethesda, Md., it must be hand-carried. He speculates that small details of experimental lab conditions may be partly to blame for other labs' trouble in reproducing his data, details such as the way mice are injected or the freeze-thaw cycle involved in shipping endostatin.

Dr. Olsen, the EntreMed consultant, thinks otherwise. He has been able to get dozens of endostatin variants to slow the proliferation of blood-vessel cells, but only slightly. He says one possibility is that some unknown contaminant was the cause of the tumor-shrinking power in mice that Dr. Folkman found -- a notion Dr. Folkman rejects, citing three proofs of purity he performed.

As for EntreMed, its chairman, John Holaday, says, "I'm not going to let delays in mouse studies hinder us in our approach, which is to test human endostatin in people." EntreMed still needs a corporate partner before it can begin such tests.

Leon Rosenberg, Bristol-Myers' former research chief, points to subjective factors that can muddy laboratory results. "You had people who worked in Judah's lab trying to tell the difference between one plus and four plus" in blood-vessel inhibition, which are minute differences, he says. "That was often in the eye of the beholder."

Similar criticism dogged a study Dr. Folkman once co-wrote on treating life-threatening birthmarks. The 1992 New England Journal of Medicine article was corrected not once but twice over the next three years because measurement was more subjective and side effects more substantial than had been stated. Says Dr. Folkman: "It could have been carelessness."

Others' Studies

As to his latest biological agents, however, Dr. Folkman says that several studies by people outside his lab should help dispel doubt about his dramatic data. One, published in Nature last July, said angiostatin enhanced the effects of radiation therapy. It was "a stunning paper," Dr. Folkman says.

But he was one of the three outsiders who "peer-reviewed" it -- and the only reviewer who had no misgivings, according to one of the study's authors. Dr. Folkman says he would never endorse a study that wasn't top notch merely to bolster his own work. His critics are "jealous," Dr. Folkman says, because of the recent publicity he has received, and he finds the doubts about his work "very depressing."

The National Cancer Institute will soon have an opportunity to study Dr. Folkman's methods firsthand, by sending in its own team of scientists to his lab. The institute, says Robert Wittes, its deputy director of extramural science, is "frustrated" that its researchers can't reproduce Dr. Folkman's "striking observations" after almost a year of trying. "We are all puzzled by this and feel we need to get to the bottom of this before we move forward," he says.

Even if Dr. Folkman doesn't find a powerful cancer drug himself, his pioneering work may lead others to effective therapies using his approach of blocking cancer's blood supply. Companies such as Bayer AG, British Biotech PLC and Agouron Pharmaceuticals Inc. are testing their own such agents in final-stage clinical trials.

Dr. Folkman's long struggle for a human-cancer breakthrough just demonstrates that pioneering science is like a "marathon," says longtime associate Jordan Gutterman at the M.D. Anderson Cancer Center. It's a race he thinks Dr. Folkman is destined to win. "His body of work is extraordinary, and my strong opinion is that it will be borne out in time."

EntreMed Transfers Rights to Endostatin & Angiostatin February 04, 2004

ROCKVILLE, MD, Feb. 4 -- EntreMed, Inc. (Nasdaq: ENMD), a biopharmaceutical company focusing on the research and development of small molecule therapeutics, today announced the transfer of all rights for its protein-based drug candidate programs, endostatin and angiostatin, in an agreement with Children's Medical Center Corporation (CMCC) in Boston and Alchemgen Therapeutics, Inc. Pursuant to the agreement, CMCC and Alchemgen continue the development of endostatin and angiostatin and bear all expenses associated with such programs, including costs that EntreMed may incur in transferring these compounds. In exchange for these programs, EntreMed receives upfront and future cash and royalty payments. Clinical patients currently receiving endostatin and angiostatin continue to be treated.

In 2003, EntreMed made a strategic decision to concentrate the Company's research and development efforts on its small molecule oncology program. The Company's activities are now directed to its Phase II clinical program and advanced preclinical pipeline.

Neil Campbell, EntreMed President and Chief Operating Officer, commented, "This agreement with Children's and Alchemgen ensures the continued commercial development of endostatin and angiostatin, while we focus our financial and scientific resources on our promising product candidate pipeline. As we enter 2004, we have significantly strengthened our financial position."

Under the terms of the three-party agreement, the Houston-based, privately-held company Alchemgen receives exclusive rights to market endostatin and angiostatin in Asia. CMCC holds the license for the rest of the world. EntreMed receives 20% of all future proceeds (e.g. upfront, milestone and royalty payments) resulting from any subsequent CMCC license outside of Asia.

About EntreMed

EntreMed, Inc. is a clinical-stage biopharmaceutical company developing therapeutics that simultaneously target the biological pathways of angiogenesis (abnormal blood vessel growth) inflammation, coagulation and/or apoptosis (programmed cell death) -- pathways associated with over 80 diseases such as cancer, blindness and atherosclerosis. The Company's clinical drug candidates, led by the small molecule Panzem®, have shown a strong safety profile with neither toxicity nor clinically significant side effects reported to date. Further, doctors have reported tumor regression and disease stabilization in some clinical patients that have received EntreMed drug candidates. The Company also has a rich pipeline of small molecule compounds in preclinical development, consisting primarily of new chemical entities based on 2-methoxyestradiol-related structures (steroidal and non-steroidal analogs) and chemically-derived small molecules from peptides -- peptidomimetics. For further information, visit EntreMed's web site at www.entremed.com.

About Alchemgen Therapeutics, Inc.

Alchemgen Therapeutics, Inc. is a research-based biopharmaceutical company dedicated to the development of compounds for cancer therapy. The Company is privately financed. For more information, please visit www.alchmgen.com

Forward Looking Statements

This release contains, and other statements that EntreMed may make may contain, forward-looking statements within the meaning of the Private Securities Litigation Reform Act with respect to the outlook for expectations for future financial or business performance, strategies, expectations and goals. Forward-looking statements are typically identified by words or phrases such as "believe," "feel," "expect," "anticipate," "intend," "outlook," "estimate," "target," "assume," "goal," "objective," "plan," "remain," "seek," "trend," and variations of such words and similar expressions, or future or conditional verbs such

as "will," "would," "should," "could," "might," "can," "may," or similar expressions. Forward-looking statements are subject to numerous assumptions, risks and uncertainties, which change over time. Forward-looking statements speak only as of the date they are made, and EntreMed assumes no duty to update forward-looking statements. Actual results could differ materially from those currently anticipated due to a number of factors, including those set forth in EntreMed's Securities and Exchange Commission filings under "Risk Factors," including risks relating to EntreMed's need for additional capital and the uncertainty of additional funding, the early stage of products under development; uncertainties relating to clinical trials; dependence on third parties; future capital needs; and risks relating to the commercialization, if any, of the Company's proposed products (such as marketing, safety, regulatory, patent, product liability, supply, competition and other risks).

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EntreMed Announces Licensing Deal With Oxford BioMedica September 09, 2003

ROCKVILLE, MD, Sep 9, 2003 -- EntreMed, Inc. (Nasdaq: ENMD) today announced the execution of an agreement granting Oxford BioMedica plc (London: OXB) exclusive worldwide rights to develop the localized delivery of two gene targets from EntreMed as a possible treatment for the loss of vision.

Under the terms of the agreement, Oxford BioMedica receives exclusive worldwide rights to use EntreMed's endostatin and angiostatin genes in the development of locally delivered gene therapy for ophthalmologic applications. Oxford BioMedica plans to utilize EntreMed's genes in its proprietary therapeutic RetinoStat™ program for the treatment of age-related macular degeneration and diabetic retinopathy. In return, EntreMed receives an initial cash payment and Oxford BioMedica common stock. Additionally, EntreMed may collect up to $10 million on the achievement of regulatory and clinical milestones. The Company may receive royalties on future worldwide sales of products resulting from the agreement. EntreMed retains rights to gene therapy applications outside of localized delivery to the eye. EntreMed also retains the rights to the endostatin and angiostatin proteins and is actively pursuing alternative commercialization of these two clinical drug candidates.

Neil Campbell, EntreMed President and Chief Operating Officer, commented, "Given Oxford BioMedica's gene therapy expertise, we are pleased that we can continue the commercialization of a gene-based endostatin and angiostatin treatment for vision loss. We are also exploring possible relationships with Oxford BioMedica in the areas of hypoxia and hypoxia-induced diseases, such as cancer, anemia and cardiovascular disease." Mr. Campbell continued, "Today's announcement is the first of several activities EntreMed has been exploring to continue the commercial development of endostatin and angiostatin and further demonstrates EntreMed's commitment to finalize our stated realignment that will allow the Company to focus solely on its small molecule programs."

Oxford BioMedica's RetinoStat™ is a LentiVector gene delivery system expressing an angiostatic gene under the control of its proprietary hypoxia response element gene switch. RetinoStat™ is currently in preclinical development.

About EntreMed
EntreMed, Inc. is a clinical-stage biopharmaceutical company developing therapeutics that simultaneously target the biological pathways of angiogenesis (abnormal blood vessel growth) inflammation, coagulation and/or apoptosis (programmed cell death) -- pathways associated with over 80 diseases such as cancer, blindness and atherosclerosis. The Company's clinical drug candidates, led by the small molecule Panzem®, have shown a strong safety profile with neither toxicity nor clinically significant side effects reported to date. Further, doctors have reported tumor regression and disease stabilization in some clinical patients that have received EntreMed drug candidates. The Company also has a rich pipeline of small molecule compounds in preclinical development, consisting primarily of new chemical entities based on 2-methoxyestradiol-related structures (steroidal and non-steroidal analogs) and chemically-derived small molecules from peptides -- peptidomimetics. For further information, visit EntreMed's web site at www.entremed.com.

About Oxford BioMedica
Oxford BioMedica (London: OXB) is a biopharmaceutical company specializing in the development of gene-based products for a range of unmet medical needs with an emphasis on new cancer products, which combine novel mechanisms of action with very low side effects, and innovative neurotherapy products, which address large and, in several areas, untapped markets. The products are all protected by multiple patents comprising a total intellectual property portfolio of some 69 patent families, which represents one of the broadest patent estates in the field. In addition to its technical research skill- base, Oxford BioMedica has in-house clinical, regulatory and manufacturing know-how. The development pipeline

includes two novel anti-cancer products in clinical trials and a gene-based treatment for Parkinson's disease, which is in late preclinical studies. Oxford BioMedica has a wholly-owned subsidiary in San Diego, USA. Oxford BioMedica has corporate collaborations with Wyeth, Intervet, Aliga Pharmaceuticals, Amersham, Arius Research and Viragen. Further information is available at http://www.oxfordbiomedica.co.uk

Forward Looking Statements

This release contains, and other statements that EntreMed may make may contain, forward-looking statements within the meaning of the Private Securities Litigation Reform Act with respect to the outlook for expectations for future financial or business performance, strategies, expectations and goals. Forward-looking statements are typically identified by words or phrases such as "believe," "feel," "expect," "anticipate," "intend," "outlook," "estimate," "target," "assume," "goal," "objective," "plan," "remain," "seek," "trend," and variations of such words and similar expressions, or future or conditional verbs such as "will," "would," "should," "could," "might," "can," "may," or similar expressions. Forward-looking statements are subject to numerous assumptions, risks and uncertainties, which change over time. Forward-looking statements speak only as of the date they are made, and EntreMed assumes no duty to update forward-looking statements. Actual results could differ materially from those currently anticipated due to a number of factors, including those set forth in EntreMed's Securities and Exchange Commission filings under "Risk Factors," including risks relating to EntreMed's need for additional capital and the uncertainty of additional funding, the early stage of products under development; uncertainties relating to clinical trials; dependence on third parties; future capital needs; and risks relating to the commercialization, if any, of the Company's proposed products (such as marketing, safety, regulatory, patent, product liability, supply, competition and other risks).

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Laboratory Investigation

Lack of antitumor activity of recombinant endostatin in a human neuroblastoma xenograft model

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Key words: angiogenesis, endostatin, neuroblastoma, pharmacokinetics, recombinant protein

Summary

Patients with metastatic neuroblastoma are rarely curable with currently available therapy, and the search for new treatment options, which include the use of inhibitors of tumor angiogenesis, is warranted. Here, we have evaluated the efficacy of one of the most promising natural inhibitors of angiogenesis described to date, endostatin, in a human neuroblastoma xenograft model in nude mice. Murine endostatin cDNA was cloned in a bacterial expression vector, expressed as a polyHis-Endostatin fusion protein and purified on Ni^{2+}-NTA beads. The *in vitro* activity of soluble endostatin was confirmed on bovine capillary endothelial cells and human umbilical vein endothelial cells. The human neuroblastoma cell line SKNAS was injected subcutaneously in the flank of nude mice and administration of the recombinant angiogenesis inhibitor started when tumors reached the size of 100 μm^3. Twenty mg/kg of recombinant precipitated endostatin or PBS was subcutaneously injected daily for 12 days. Serum endostatin levels were measured using a competitive enzyme immunoassay. Tumor growth was only slowed down in endostatin-treated mice when compared to control mice, and no statistically significant difference in serum levels of endostatin was observed between endostatin-treated and control groups. The lack of correlation between serum concentration and tumor response raises concern regarding the mechanism of action of endostatin.

Introduction

Neuroblastoma is the most common extracranial solid tumor in children younger than 5 years old. Although minimal treatment is sufficient for many children with localized tumors, metastatic neuroblastoma is rarely curable, even with high-dose chemotherapy followed by bone marrow transplantation [1]. New therapeutic approaches are therefore warranted.

Recent advances on angiogenesis research have shed new light on the growth and metastasis of solid tumors, and have allowed the definition of a new paradigm for cancer treatment [2]. Tumor neovascularization is a complex process tightly controlled by negative and positive regulators, and it has been suggested that the overall balance between these factors is responsible for the angiogenic phenotype of cancerous tumor [3]. These studies have highlighted the dramatic therapeutic potential of natural inhibitors of angiogenesis, which were found capable of maintaining tumors in a state of dormancy [4]. Among the known natural inhibitors of angiogenesis, one of the most potent is endostatin. Endostatin was purified from conditioned media of a murine endothelioma cell line and was found to be identical to the C-terminal fragment of collagen XVIII [5]. *In vitro*, recombinant murine endostatin inhibited endothelial cell proliferation in a dose-dependent manner, starting at 100 ng/ml with a maximum effect of 66% inhibition being reached at 600 ng/ml [5]. When administered subcutaneously as a precipitated protein, endostatin caused the regression of several experimental tumors and was able to induce a prolonged tumor

dormancy after two to six repeated administrations [6]. O'Reilly and colleagues have proposed that the injected, non-refolded endostatin protein acts as a subcutaneous depot that results in slow protein release over a 24–48 h period. Although these studies have shown the therapeutic activity of precipitated endostatin in syngenic murine tumor models, no data have been made available regarding the serum concentrations of endostatin attained using this delivery technique.

Here, we have evaluated the efficacy of precipitated recombinant endostatin in a murine model of human neuroblastoma in nude mice, and quantified serum levels of recombinant endostatin obtained using this procedure.

Materials and methods

Vector construction

The murine endostatin cDNA was derived from the mouse collagen XVIII cDNA, which was kindly provided by Dr. B.R. Olsen (Harvard Medical School, Boston, MA). The KA#135 plasmid is described elsewhere [7]. PQE 40 expression vector was obtained from Qiagen (Qiagen GmbH, Hilden, Germany).

The endostatin expression plasmid was constructed by isolating the 570 bp HindIII-Xho I fragment (containing the 552 base pair C-terminal fragment of murine collagen XVIII) from the KA#135 plasmid and ligating it to PQE 40 plasmid digested with BamH I and Sal I. This was done by triple ligation with a linker/adapter between BamH I and Hind III restriction sites. The resulting plasmid was named KAL#19.

Production and purification of His-tagged murine endostatin

Protein production and purification were performed following the protocol described by O'Reilly et al. [5]. The bacteria used was the *E. coli* strain MP15 (Qiagen GmbH, Hilden, Germany), which contains the low copy plasmid pREP4 constitutively expressing the lac repressor protein encoded by the lacI gene. Ni^{2+}-NTA beads were purchased from Qiagen. Purification under denaturing conditions was done according to the manufacturer's instructions (Qiaexpressionist handbook, Qiagen).

After elution from Ni^{2+}-NTA beads, the solution was dialyzed against PBS at 4°C for approximately 72 h using Spectrum spectra/pore membrane MWCO 6000–8000 kDa (Spectrum Laboratories Inc., Laguna Hills, California, USA). About 90% of the amount of endostatin precipitated during this step, and then was collected by centrifugation. The precipitated fraction was resuspended in PBS and its concentration assessed by weighing a desiccated aliquot. Precipitated endostatin was adjusted to a concentration of 3 mg/ml, aliquoted in 300 µl fractions, and then used for the *in vivo* experiments. The supernatant containing the soluble fraction was concentrated using Millipore filter Centricon plus-20 and Centricon YM-10 (Millipore Corporation, Bedford, Massachusetts, USA) to a concentration of 50 µg/ml, as assessed by the Accucyte Murine Endostatin Kit (Cytimmune Sciences Inc., College Park, Maryland, USA). The soluble fraction was used for the *in vitro* endothelial cell proliferation assays. All recombinant proteins were kept frozen at −20°C.

The 6 × His-tagged prostate-specific antigen (PSA) used as a negative control for the Western analysis was produced and purified using identical methods as for murine endostatin [5].

Cell culture

Bovine capillary endothelial cells (BCE) obtained from adrenal glands were grown in DMEM supplemented with 10% fetal calf serum, gentamycin (20 µg/ml), penicillin (20 UI/ml), streptomycin (20 µg/ml) and nystatin (10 UI/ml). Human umbilical vein endothelial cells (HUVEC) purchased from Clonetics (Walkersville, USA) were grown in DMEM supplemented with 10% fetal calf serum, gentamycin (10 µg/ml) and streptomycin (10 µg/ml). The human neuroblastoma cell line SKNAS (ATCC CRL-2137) was maintained in RPMI supplemented with 10% calf serum, 20 UI/ml penicillin, 20 µg/ml streptomycin and 10 µM L-glutamine.

Cell proliferation assay

The antiproliferative effect of the soluble fraction of His-tagged recombinant murine endostatin was tested on BCE, HUVEC and SKNAS cells.

For proliferation assays with BCE and SKNAS, cells were plated at a density of 5000 cells/well (BCE) or 2000 cells/well (SKNAS) in 16-well plates. After a 4 h incubation, the medium was replaced with fresh medium containing 1 ng/ml of βFGF and the test sample applied. After 72 h, cells were dispersed in trypsin

and counted with a hemocytometer. Each experiment was done in triplicate.

For proliferation assay with HUVEC, cells were plated at a density of 2000 cells/well in a 96-well plate. After a 2 h incubation, the medium was replaced with fresh medium containing 10 ng/ml of βFGF, increasing concentrations of endostatin and 1.5 μCi/ml of ^{3}H-thymidine. The cells were incubated for 24 h, washed, solubilised, and then cell-associated radioactivity was determined using a liquid scintillation counter.

Gel electrophoresis and immunoblotting

Recombinant proteins were analyzed on SDS–PAGE gels. The gels were stained with Coumassie blue. For Western blot analysis, the proteins were transferred onto nitrocellulose membrane. Membranes were incubated with a polyclonal goat–anti-mouse endostatin antibody (R&D Systems, Abingdon, UK) diluted 1:1000 in PBS. Membranes were then incubated with rabbit–anti-goat IgG HRP-labelled secondary antibody (Dako, Glostrup, Denmark) diluted 1:2000 in PBS. Proteins were visualized using the ECL+ system (Amersham, Paris, France). Recombinant PSA was used as a negative control in the Western blot analysis.

Endostatin immunoassays

Recombinant soluble endostatin concentrations and serum and urine endostatin levels were measured using a commercially available competitive enzyme immunoassay, the Accucyte Murine Endostatin Kit from Cytimmune (Cytimmune Sciences Inc., College Park, Maryland, USA). The reaction was performed according to the manufacturers instructions.

Animal experimentation

Six-week-old male 'nude' mice were purchased from Iffa-Credo (Iffa-Credo S.A., L'Arbresle, France). The mice were maintained in appropriate facilities under pathogen-free conditions. The mice received 10^6 SKNAS cells in 200 μl PBS injected subcutaneously in the right flank. Tumors were visible after 10–15 days; treatment was started when they reached the size of 100 mm^3. Tumor volume was determined using a standard formula [6]. Only the number of aliquots of recombinant murine endostatin necessary for one injection was thawed at the last moment. The suspension was homogenized in a 1 ml syringe, and then 600 μg of recombinant murine endostatin (20 mg/kg) in 200 μl was injected subcutaneously in the back of the mice, between the scapula, using a 25 G × 1/2″ needle (Terumo, Leuven, Belgium). Treatment was repeated every day for 12 days. Tumor volume was measured every 4 days. After 12 days of treatment, blood was taken by heart puncture for assessment of serum endostatin levels and the mice were sacrificed.

For pharmacokinetics experiments, six mice were injected with a single dose of 20 mg/kg of precipitated recombinant murine endostatin. Blood was taken by retro-ocular puncture before injection and 4, 8, 24, 48, and 72 h after injection (only two or three mice were punctured each time due to repeated sampling). The urine of three of the mice was collected every 24 h, from 24 h before injection to 96 h after injection.

Statistical analysis

In vitro and *in vivo* data were expressed as means +/− standard deviations (s.d.). *In vivo* data represent average tumor volumes from three independent experiments. A non-parametric test, the Mann–Whitney *U*-test, was used for all statistical analyses.

Results

Vector construction for bacterial expression of murine endostatin

By subcloning the 552 bp fragment encoding the C-terminal portion of murine collagen XVIII into PQE 40, we created an artificial gene coding for a polyHis-Endostatin fusion protein. The predicted N-end of this 6×His-tagged endostatin included the following amino acid: MRGSHHHHHHGSGGKLHTHQ ... , HTHQ being the N-end of the mouse endostatin [5]. In the resulting expression vector KAL#19, the 6 × His-tagged endostatin is under the transcriptional control of the phage T5 promoter and two lac operator sequences. KAL#19 was sequenced on both strands and transformed in the *E. coli* strain MP15, which contains the low copy plasmid pREP4 constitutively expressing the lac repressor protein encoded by the lac I gene.

Purification and characterization of recombinant murine endostatin

The expression of 6 × His-tagged murine endostatin from KAL#19 was almost completely repressed in the

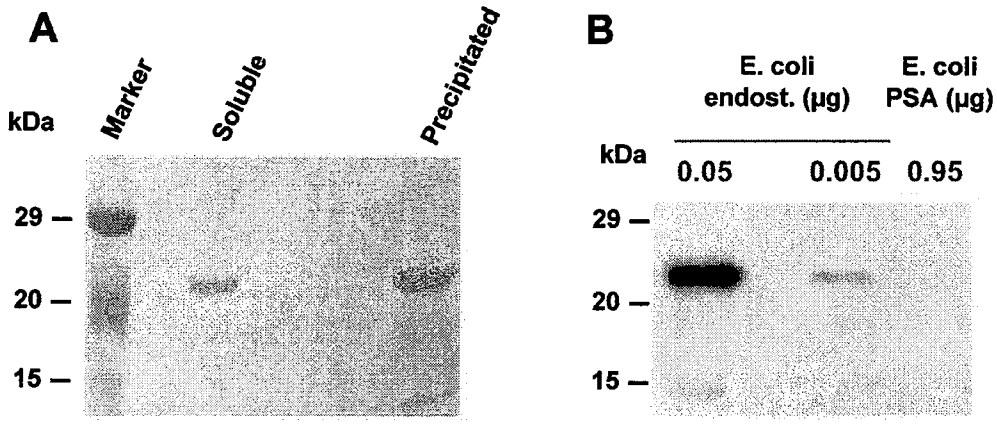

Figure 1. (A) SDS–PAGE analysis of recombinant murine endostatin. Twenty-five micrograms of concentrated soluble protein fraction and precipitated protein fraction obtained after dialysis were analyzed by electrophoresis on a 15% SDS polyacrylamide gel and detected by Coumassie blue staining. The major band has a molecular weight of 24 kDa, in agreement with the theoretical size of endostatin. (B) Western blot analysis of recombinant murine endostatin. After electrophoresis, recombinant endostatin and recombinant prostate-specific antigen (MW: 25 kDa) were immunoblotted, and the membrane was incubated with a polyclonal antibody directed against endostatin. The molecular size markers are indicated. The lower band migrating at approximately 13 kDa reacts with the antibody and may represent a smaller fragment of endostatin.

absence of inducer, whereas it was greatly increased in the presence of IPTG (data not shown). The recombinant protein was purified under denaturing conditions and subjected to dialysis (Figure 1A). As described by O'Reilly et al. [5], most of the recombinant endostatin precipitated during dialysis. The amount of polyHis-Endostatin produced from 1 l of culture medium using this procedure was 7–10 mg of precipitated protein and up to 500 µg of soluble protein. SDS–PAGE analysis showed a protein with a size consistent with that of 6 × His-tagged murine endostatin (Figure 1A). Endostatin was estimated to be about 90% pure (Figure 1A).

The integrity of the recombinant protein was confirmed by Western blot analysis using a polyclonal antibody directed against murine endostatin (Figure 1B). A recombinant 6 × His-tagged PSA protein, produced and purified in the same way as endostatin, was used as a negative control (Figure 1B). The lower band migrating at approximately 13 kDa on the same lane as endostatin is thought to represent a fragment of endostatin for two reasons. This band reacts with the polyclonal antibody, and no band of similar size is detected in the PSA lane, which would be the case if this band represented a co-purified bacterial protein (Figure 1B).

Biological activities of recombinant murine endostatin

Recombinant soluble murine endostatin was tested for its ability to inhibit cell proliferation *in vitro*. BCE proliferation was inhibited in a dose-dependent manner at a half-maximal dose of approximately 275 ng/ml (Figure 2A). By contrast, the neuroblastoma cell line SKNAS was not inhibited at 2000 ng/ml (Figure 2A). HUVEC cell proliferation was inhibited by 70% at a concentration of 500 ng/ml (Figure 2B).

In vivo *activity of recombinant murine endostatin in a xenograft model of human neuroblastoma*

We investigated whether recombinant murine endostatin could inhibit tumor growth in a model of human neuroblastoma xenografted into nude mice. Treatment was started when tumor volumes were approximately 100 mm^3. A total of 18 mice in three successive experiments were treated with daily administrations of 20 mg/kg recombinant murine endostatin and compared to 15 mice injected with PBS alone. Tumor growth was inhibited by approximately 56% in endostatin-treated mice when compared to control mice (Figure 3). However, due to large standard

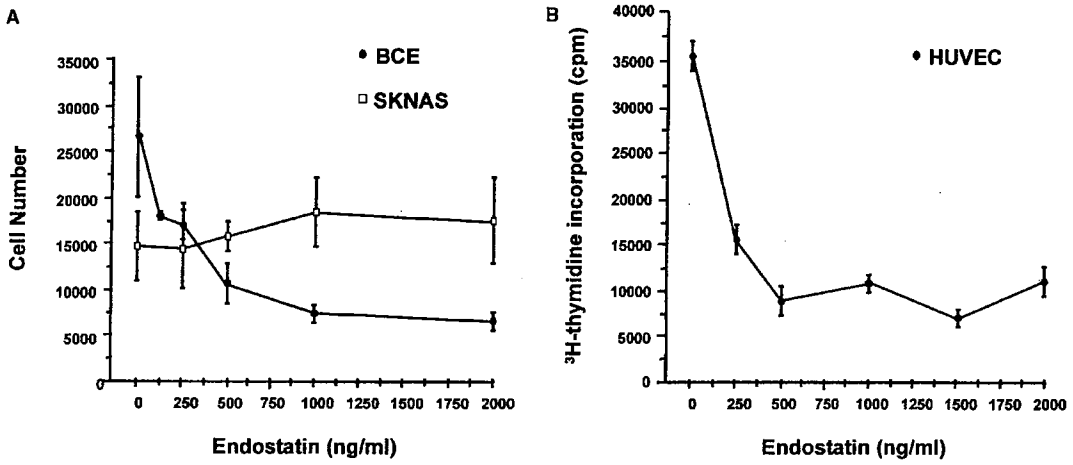

Figure 2. (A) Inhibition of BCE and SKNAS cell proliferation by recombinant murine endostatin in a 72 h proliferation assay. BCE cells were plated at a density of 5000 cells/well and SKNAS cells at a density of 2000 cells/well. Samples of recombinant endostatin were applied after 4 h together with 1 ng/ml of βFGF. Each value represents the mean +/− s.d. of triplicate cultures. (B) Recombinant murine endostatin was tested for its ability to inhibit ^{3}H-thymidine incorporation in HUVEC cells stimulated by βFGF (10 ng/ml). HUVEC cells were plated at a density of 2000 cells/well, and then βFGF and increasing concentrations of recombinant endostatin were added. The amount of ^{3}H-thymidine incorporated was determined after 24 h. The experiment was repeated 6 times; each value represents the mean +/− s.d.

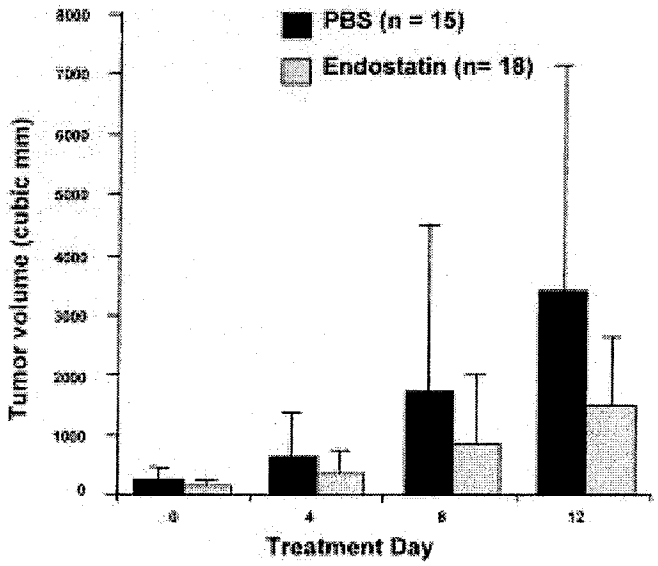

Figure 3. Growth retardation of SKNAS neuroblastoma in nude mice. Six-week-old male nude mice were injected subcutaneously with 10^6 SKNAS cells. Treatment with PBS ($n = 15$) or recombinant precipitated endostatin 20 mg/kg/day ($n = 18$) was started after tumors had reached 100 mm^3. Tumor growth in the endostatin-treated group was inhibited by 56% but the difference did not reach statistical significance (Mann–Whitney U-test; two-sided $p = 0.1$). Each bar represents the mean +/− s.d.

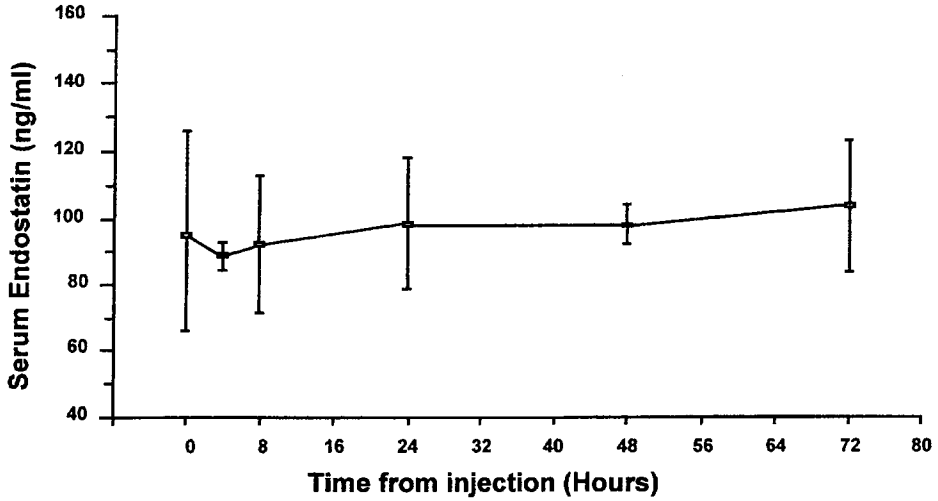

Figure 4. Serum levels of endostatin after a single subcutaneous injection of 20 mg/kg (600 μg) of recombinant precipitated endostatin. At each time, blood samples were collected by retro-ocular puncture of two or three mice from a group of six mice. Each bar represents the mean serum concentration +/− s.d.

deviations in both treated and control groups, these results were not statistically significant (Mann–Whitney *U*-test; two-sided $p = 0.1$).

Pharmacokinetics of recombinant murine endostatin

In order to estimate the amount of endostatin reaching the vascular compartment after subcutaneous injection of precipitated protein, we assessed its serum and urine concentrations after a single injection and at the end of a 12 day experiment with daily injections.

After a single injection of 20 mg/kg (600 μg) of recombinant endostatin (see Figure 4), we could not detect any rise in the serum concentration of endostatin over control mice, whose serum levels were stable around 100 ng/ml for 4 days. At the same time, there was a trend for a higher urine secretion of endostatin between 24 and 72 h post injection (baseline 30 ng/24 h, 58 ng/24 h from h 24 to h 48 and 64 ng/24 h between h 48 and h 72). Nevertheless, these differences were not statistically significant, and the amount of endostatin excreted in the urine in excess of baseline secretion during the 3 days following 600 μg endostatin injection was only 60 ng. At the end of a 12 day experiment, serum endostatin concentrations in four control mice (129+/−16 ng/ml) and in four endostatin-treated mice (117+/−16 ng/ml) could not be distinguished.

Discussion

In the present study, we have investigated the ability of recombinant murine endostatin to inhibit tumor growth in an experimental model of human neuroblastoma in nude mice.

To date, six published studies have described the treatment of tumor-bearing mice with recombinant endostatin. Three of them report a complete regression of established syngenic tumors in C57BL/6 mice using precipitated recombinant endostatin produced in *E. coli* [5,6,8]. In these experiments, treatment was started when tumor volumes reached 250–400 mm^3. Two more studies, which made use of soluble recombinant endostatin produced in yeast or in 293 cells and tested in a renal cell carcinoma xenograft model in nude mice, showed merely a growth retardation in the endostatin-treated group [9] or a maximum tumor regression of 40% with a resumption of tumor growth after 2 weeks [10]. In one study [11], an immunoglobulin G Fc fragment/endostatin fusion protein (mFc-mEndostatin) was utilized; in the supplementary material section (www.sciencemag.org/feature/data/990055.shl), the authors reported, as an unpublished observation from O'Reilly and Javaherian, a very high efficacy of this fusion protein in the Lewis Lung Carcinoma transplant model in C57BL/6 mice. By contrast, the mFc-mEndostatin did not show any efficacy by itself in the

RIPI-Tag2 transgenic mouse model when spontaneous pancreatic islet cell tumors were treated after they had reached a mean tumor burden of 77 mm^3 [11]. More recently, precipitated rat endostatin was shown to be highly effective on a carcinogen-induced rat mammary tumor model [12].

Accordingly, it appears that the best results published so far with endostatin have been obtained with the original method of bacterial production and subcutaneous injection of the precipitated protein. In this method, six histidine residues were fused to the N-terminus of endostatin, and then the resulting artificial protein was purified by standard Ni^2-NTA affinity. The design of our bacterial expression vector is only slightly different from the vector used by O'Reilly et al. [5,8], and the recombinant protein is identical but for its N-terminus.. Despite this small difference, the purification procedure resulted in a comparable yield of protein production. As previously described [5,12], most of the endostatin precipitated during dialysis against PBS, although a small percentage spontaneously solubilized. The concentration of this solubilized fraction could be estimated by the Accucyte Murine Endostatin test from Cytimmune and was used for *in vitro* BCE and HUVEC assays, thereby confirming its biological activity.

In contrast to the *in vitro* results, our *in vivo* experiments did not show any significant activity of endostatin in our model of human neuroblastoma in nude mice. Tumor growth was only slowed by 56% in the endostatin-treated group, and the difference after 12 days of treatment did not reach statistical significance.

In order to estimate the pharmacological distribution of endostatin after subcutaneous injection of the precipitated protein, we performed a series of serum dosage using a competitive enzyme immunoassay. We could not detect any rise in serum endostatin levels after either a single injection of 600 μg of endostatin or 12 days of continuous treatment, which represented a total dose 7.2 mg of endostatin injected subcutaneously. Urine analysis of treated mice did not show any rise in endostatin excretion in relation with the amount of injected protein.

The most puzzling finding of our study is that, despite a trend for efficacy in the endostatin-treated group, we could not find a substantial rise in serum endostatin levels in the treated mice. Three hypotheses may explain these data. The first one is the possible strong binding of endostatin to endothelial cells, which would thereby prevent a rise in serum levels despite a high total 'load' along the vascular lining. The amount of endostatin administered to the mice (600 μg per injection), with respect to the weight of the animal (30 g) and the basal endostatin serum concentration (100 ng/ml), are not in favor of this hypothesis as the sole explanation for the total absence of rise in serum or urine endostatin. The second hypothesis is that the protein released from the subcutaneous depot may not properly refold and would, therefore, not be detectable by the Accucyte Murine Endostatin test. This hypothesis fits with experimental data suggesting a relation between endostatin conformation and its efficacy [8,10,13]. Finally, there may not be any significant release of endostatin from the subcutaneous depot until local degradation of the recombinant protein. In the second and third hypotheses, one could hypothesize that only a small amount of active protein reached the bloodstream and may have been responsible for the anti-tumoral activity observed in previous studies. It is difficult, nevertheless, to understand how an imperceptible rise in endostatin activity over pre-existing endogenous background can be responsible for the dramatic anti-tumor activity reported *in vivo*.

In this study, we failed to observe significant antitumor activity of recombinant endostatin in a xenograft model of human neuroblastoma. Our results and those reported by O'Reilly et al. [5] cannot rule out that a contamination by other factors produced in *E. coli*, such as endotoxin, is in fact responsible for the *in vitro* activity of recombinant endostatin in BCE assays. Specific biological modifications of the unrefolded protein and/or contamination may account in part for the reported anti-tumor activity. This could explain the important variation in efficacy observed in different animal models.

Acknowledgements

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NEWS FOCUS

CANCER THERAPY: Setbacks for Endostatin

Eliot Marshall

Harvard University's Judah Folkman electrified cancer researchers 5 years ago when he and his colleagues reported on a new compound that could shrink tumors in mice virtually to nothing by cutting off the blood supply to tumors, rather than by poisoning patients with toxic drugs. Now, as clinical trials of the widely heralded cancer treatment endostatin are about to be expanded, two groups report that they couldn't get it to work. Although these papers are not the first to raise questions about endostatin, they are among the most pointed.

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Endostatin maybe not so good..?

Posted By: **Tina T**
Date: Sunday, 31 March 2002, at 8:45 a.m.

Hello, I caught this today
Tina

Ex-colleague of Folkman to publish negative endostatin results

18 March 2002 12:43 EST
by Apoorva Mandavilli, BioMedNet News

The anti-angiogenesis factor endostatin, which entered clinical cancer trials in record time after being propelled into the public attention in 1998 by a glowing front-page article in the New York Times, is under serious challenge again. Two new papers to be published next month in Molecular Therapy echo earlier reports from scientists who could not replicate claims by Harvard University's Judah Folkman that endostatin shrinks tumors by cutting off their blood supply.

According to a report in BioMedNet News today, opinion remains sharply divided in the specialist community about whether Folkman has observed a true biological phenomenon, because endostatin still lacks a well-defined mechanism of anti-tumor action and noteworthy attempts to replicate it have failed. The situation has prompted Molecular Therapy to take the unusual step of publishing negative results.

The new studies tried several approaches to detect a measurable effect with the protein: Transfecting the gene for a soluble form of endostatin into mice, and even injecting it directly into the bloodstream. Although levels of endostatin surged to 750% normal in the gene-therapy experiment, neither strategy showed any effect on either blood vessel growth or tumor size.

"We could not see an effect of endostatin any way we tried," said Philippe Leboulch of Harvard University and the Massachusetts Institute of Technology, who carried out the research with Connie Eaves of the Terry Fox Laboratory in Vancouver and others. "It's important for the scientists and the public to know this," he added. Leboulch has collaborated with Folkman in the past.

Melinda Hollingshead, a researcher at the US National Cancer Institute, says that researchers launched clinical trials of endostatin under a "great deal of pressure," after a 1998 article in the New York Times lauded its anti-cancer potential, and this raised "a lot of false hope." Hollingshead is one of several other scientists whose lab has been unable to replicate Folkman's work.

Folkman tells BioMedNet News that although the papers are well done, they don't contradict his own results or any of 200-odd papers that substantiate them. "Something weird" may be going on that prevents the treatment from working when introduced via gene therapy, while it is effective injected as a protein, he adds. In the ongoing clinical trials involving some 180 patients so far, Folkman says, results have been "impressive" and some patients have shown tumor regression.

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"It's turned out to be a surprisingly good drug," he added. The clinical results will be published soon, in abstract form.

The negative results in mice don't prove that Folkman is wrong about endostatin, says Leboulch, who has collaborated with Folkman in the past. But he added "it's important for Folkman to recognize that one should look at different aspects, results that are not positive," he said. "Otherwise, it's too dangerous."

Whatever the clinical results, Hollingshead says, endostatin will not be accepted as mainstream cancer therapy until studies have clarified its mechanisms and resolved the inconsistencies between Folkman's labs and others. The scientific controversy over endostatin, she said, "has not even begun to be resolved."

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555 East Wells Street, Suite 1100, Milwaukee, WI 53202 info@asgt.org

ASGT Press Release

For Immediate Release
March 18, 2002

Contact: Fintan R. Steele, Ph.D.
Fax: 646-935-3742

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On the cover: Endostatin Enigma

It is not often that scientific journals publish negative results. However, if the experiments are well-performed and have an immediate and direct relation to medical research, such data are important to present. In this issue of Molecular Therapy, two papers raise a series of troubling questions about the use of endostatin, a highly trumpeted antiangiogenic molecule, in cancer treatment.

Philippe Leboulch of Harvard and the Massachusetts Institute of Technology and Connie Eaves of the Terry Fox Laboratory in Vancouver led two groups of researchers who rigorously examined gene therapy approaches to providing efficacious levels of endostatin in mouse models of different forms of cancer. Despite attaining high circulating levels of functional endostatin through the transduction of hematopoietic stem cells with the gene, the researchers did not see any significant anti-tumor effects. Furthermore, the researchers were unable to replicate previously published work that suggested efficacy in mouse models. Given that endostatin clinical trials in humans are underway based in large part on these prior publications, these articles highlight not only the question of adequate preclinical models for testing this molecule in humans, but also reveal the difficult questions that face any research initiative that seeks to move from the lab bench to the clinic.

A commentary explaining the position of the journal in publishing these papers and examining some of the questions raised accompanies these papers in this issue.

Pawliuk, R., et al. (2002). Continuous intravascular secretion of endostatin in mice from transduced hematopoietic stem cells. Mol. Ther. 5:345-351.

Eisterer, W., et al. (2002). Unfulfilled promise of endostatin in a gene therapy-xenotransplant model of human acute lymhocytic leukemia. Mol. Ther. 5: 352-359.

Steele, F. R. (2002). Can "Negative" Be Positive? (Commentary). Mol. Ther. 5: 338-339.

Targeting Cystic Fibrosis I: Respiratory treatment

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Continuous Intravascular Secretion of Endostatin in Mice from Transduced Hematopoietic Stem Cells

Robert Pawliuk,[1,2,*] Thomas Bachelot,[1,*] Omar Zurkiya,[1] Anna Eriksson,[3] Yihai Cao,[3] and Philippe Leboulch[1,2,4,†]

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Endostatin, a 20-kDa carboxy-terminal fragment of collagen XVIII, is the leading member of a class of physiologic inhibitors of angiogenesis with potent antitumor activity. Repeated subcutaneous administration of recombinant endostatin in mice led to permanent regression of established tumors to a microscopic dormant state and prompted the initiation of human clinical trials. However, a discrepancy remained unresolved: sustained tumor regression has only been observed with a non-soluble, precipitated form of recombinant endostatin produced in bacteria. To shed light on this question and establish a model of systemic anti-angiogenic gene therapy of cancer that may surmount obstacles in protein production and delivery, we transduced murine hematopoietic stem cells with a retrovirus encoding a secretable form of endostatin. Despite continuous, high-level secretion of endostatin in the vasculature of all transplanted mice, we detected neither inhibition of *in vivo* neoangiogenesis nor antitumor activity. Resolution of this paradox may come from human trials of endostatin now underway.

Key Words: endostatin, anti-angiogenesis, hematopoietic stem cells, retroviral vector, bone marrow transplantation

INTRODUCTION

Although tumor-associated angiogenesis has long been recognized as a key process in cancer progression and metastasis [1,2], the identification of potent angiogenesis inhibitors has remained rather elusive. The discovery of a group of physiological inhibitors that are generated by protease-mediated cleavage of extracellular proteins has raised considerable interest [3]. Among them, endostatin, a 20-kDa carboxy-terminal fragment of collagen XVIII, is especially potent at inhibiting the growth of various murine tumors and their metastases in animal models [4]. Furthermore, endostatin has the remarkable property in cycled therapy of inducing the regression of established tumors to a microscopic dormant state, even after prolonged discontinuation of treatment [5]. These unique properties have ushered the initiation of human clinical trials [6]. However, complete inhibition of tumor growth and tumor regression have only been reported so far with the non-soluble, precipitated form of recombinant endostatin produced in *Escherichia coli* [4,5,7–12]. It was argued that precipitated endostatin injected subcutaneously acts as a depot for slow release of the active protein in the vasculature [4]. Because the non-soluble, precipitated form of endostatin is not suitable for human clinical trials, a soluble version of the human protein was produced in yeast [12]. Although endostatin produced in yeast or mammalian cells was able to inhibit the growth of tumors, it was unable to regress established tumors [8–12] and their residual activity remained difficult to obtain in large quantities [13].

On theoretical grounds, systemic anti-angiogenic gene therapy may overcome these difficulties as well as the requirement for repeated, long-term protein administration by providing sustained therapeutic levels of endostatin in the serum [14]. So far, however, studies aimed at preventing tumor growth by the injection of plasmid DNA (either naked or complexed in liposome formulations) or recombinant adenoviruses encoding either human or murine endostatin have shown only modest inhibition of tumor growth and no tumor regression [15–27]. An important question is whether the observed discrepancy between

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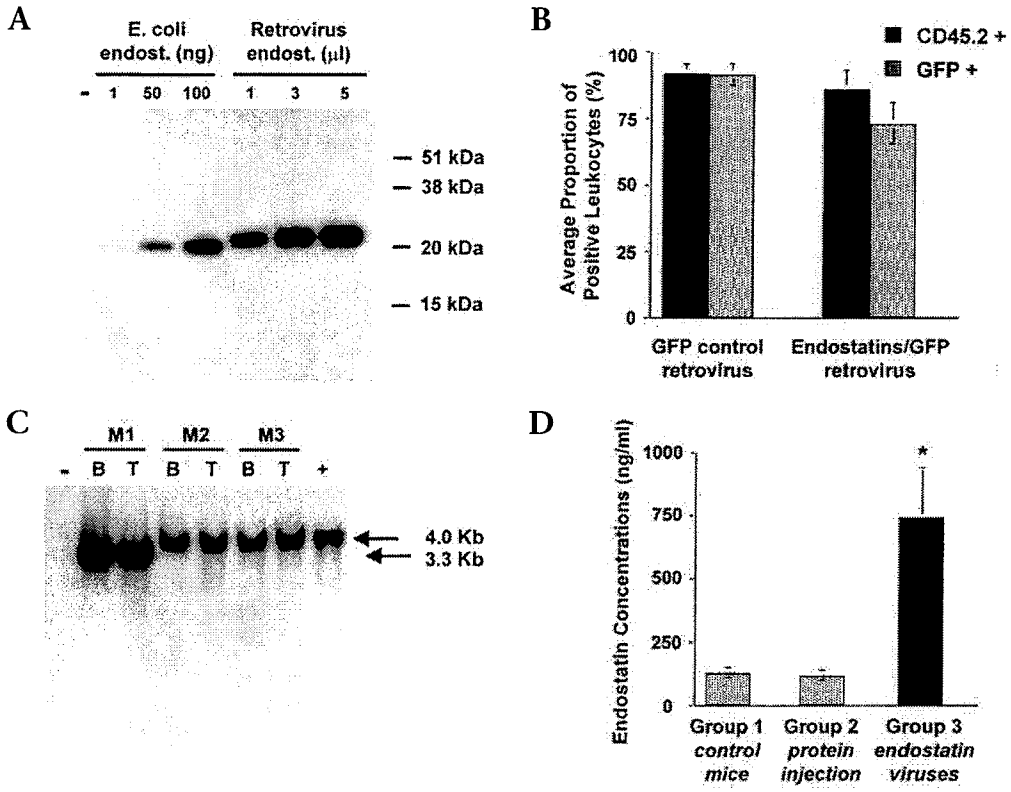

FIG. 1. Analyses of recombinant endostatin production, reconstitution of transplanted mice with donor cells, and proviral stability. (A) Detection of secreted murine endostatin from retrovirally transduced 293T cells by western blot analysis with specific polyclonal antibody. Left lane (–), supernatant from cells transduced with MSCV[GFP] control vector; middle lanes, various quantities of recombinant His-tagged murine endostatin produced in *E. coli*; right lanes, various volumes of heparin Sepharose purified murine endostatin obtained from supernatants of cells transduced with MSCV[FLAG-endostatin/GFP] virus; right margin, molecular size markers. As previously observed, endostatin expressed in mammalian cells migrates slightly slower than bacteria-produced endostatin, presumably because of differences in glycosylation [42]. (B) Quantification of the extent of reconstitution of transplanted mice with donor derived cells, on the basis of expression of the CD45.2 allele, and of the proportion of GFP+ cells among donor-derived cells, 3 months post-transplantation. Blood leukocytes were analyzed by FACS for both CD45.2 expression and GFP fluorescence. Because no statistical difference was observed among mice for MSCV[endostatin/GFP] ($n = 5$) and MSCV[FLAG-endostatin/GFP] viruses ($n = 13$), data were grouped ($n = 18$). For MSCV[GFP] control, $n = 10$. Error bars represent standard deviations. (C) Detection of intact proviruses by Southern blot analysis of bone marrow (B) and thymus (T) genomic DNA from representative mice transplanted 4 months earlier with MSCV[GFP] control (M1) or MSCV[FLAG-endostatin/GFP] (M2-3) transduced marrow. (–), Bone marrow from a non-transplanted mouse; (+), NIH3T3 cell clone with two copies of MSCV[FLAG-endostatin/GFP] provirus. DNA was digested with *Sac*I. GFP was used as a probe. (D) Quantification of serum levels of murine endostatin in mice by ELISA. Group 1, control mice transplanted with MSCV[GFP] transduced marrow ($n = 10$); group 2, mice after a 12-day regimen of daily injections of bacteria produced His-tagged murine endostatin (20 mg/kg/day) administered as a purified precipitate as described [4] ($n = 4$); group 3, mice transplanted with marrow transduced with the endostatin expressing viruses ($n = 18$). Because no statistical difference was observed among mice for MSCV[endostatin/GFP] ($n = 5$) and MSCV[FLAG-endostatin/GFP] viruses ($n = 13$), data were grouped ($n = 18$). Error bars represent standard deviations. *$P < 0.001$.

gene transfer and protein studies is due to the production of suboptimal levels of endostatin and/or transient expression following gene transfer.

To investigate whether sustained, high-level release of a soluble form of endostatin can result in tumor regression and to establish a model of systemic gene therapy of cancer through angiogenesis inhibition, we set out to obtain continuous intravascular release of endostatin by retrovirus-mediated gene transfer of a secretable form of murine endostatin into hematopoietic stem cells, followed by engraftment of syngeneic mouse recipients. We constructed two bicistronic vectors based on the murine stem cell virus (MSCV) backbone. These vectors contained cDNAs encoding secretable, FLAG-tagged or non-tagged forms of endostatin. In addition vectors also contained the gene encoding the enhanced green fluorescence protein (GFP) linked in *cis* to the internal ribosomal entry site (IRES) from the encephalomyocarditis virus 3′ of the endostatin cDNA to enable the preselection of retrovirally transduced bone marrow cells. Transplantation of retrovirally transduced, GFP+ fluorescence activated cell sorted (FACS) bone marrow cells into recipient mice resulted in stable, long-term reconstitution of all hematopoietic lineages with GFP+ cells and continuous, high-level expression of endostatin in the serum of all mice. Despite this, neither inhibition of *in vivo* neoangiogenesis nor antitumor

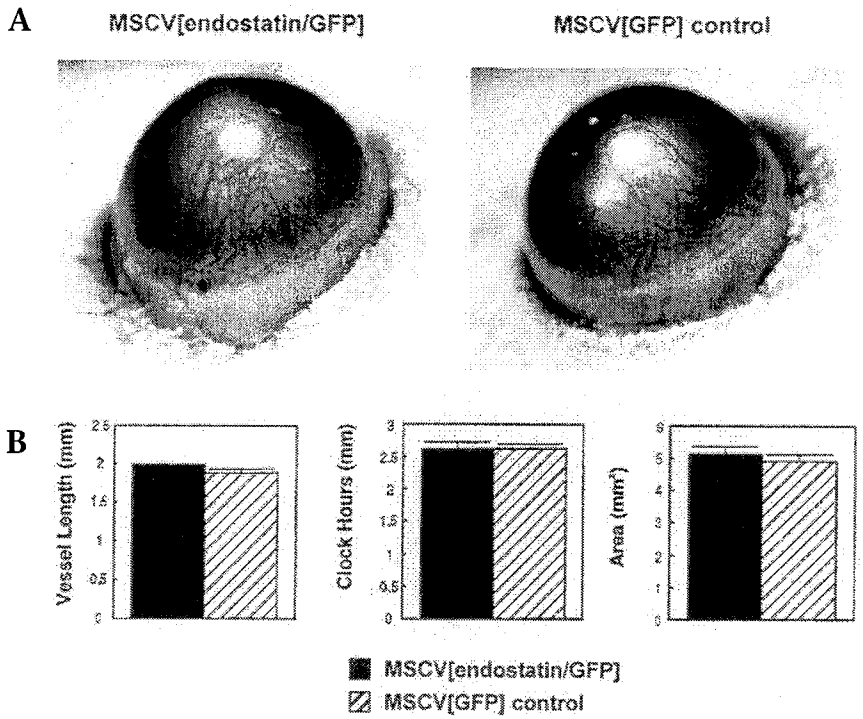

FIG. 2. Lack of systemic inhibition of neoangiogenesis in recipient mice 3 months post-transplantation, as assessed by the corneal micropocket assay with implantation of a FGF-2 pellet. (A) Corneal vascularization achieved in representative recipients of MSCV[endostatin/GFP] (left) or MSCV[GFP] (right) transduced marrow. (B) Quantification of the length (left), clock hours (middle), and area (right) of corneal vessels in mice transplanted with either MSCV[endostatin/GFP] (n = 5) or MSCV[GFP] (n = 4) transduced marrow and subjected to the corneal micropocket assay. Error bars represent standard deviations.

activity was detected. These data demonstrate that sustained intravascular delivery of a soluble form of endostatin does not recapitulate results obtained with non-soluble, bacteria-derived endostatin.

RESULTS

Construction and Characterization of Retroviral Vectors

Originally, endostatin-induced tumor regression was demonstrated with a recombinant protein in which 10 plasmid-encoded amino acids followed by 6 histidine residues (6 × His) were fused to the amino-terminal amino acids of endogenous endostatin [4]. Other studies have used either non-tagged or His– influenza virus hemagglutinin A (HA)- or murine immunoglobulin γ-2A chain (Fc)-tagged endostatin in the same N-terminal position [7–12,28]. We constructed two vectors with or without the FLAG tag, referred to as MSCV[FLAG-endostatin/GFP] and MSCV[endostatin/GFP], respectively, to gather information on both instances. A vector that only expresses GFP, referred to as MSCV[GFP], was used as a gene transfer control.

We generated recombinant retroviruses using the ecotropic packaging cell line BOSC23, with titers greater than 5×10^5 infectious units/ml on NIH3T3 cells. We documented stable proviral transfer by Southern blot analysis of transduced cells (data not shown). To verify the integrity of the secreted proteins and quantify their production, we performed western blot analysis of transduced cell supernatants with a polyclonal antibody specific for murine endostatin (Fig. 1A). Microsequencing of purified secreted endostatin confirmed its authenticity. Proliferation of bovine endothelial cells (BCE) was inhibited by 15–25% in the presence of supernatants of MSCV[FLAG-endostatin/GFP] or MSCV[endostatin/GFP] transduced 293T cells as compared with MSCV[GFP] transduced 293T cells (data not shown).

Preselection and Transplantation of Transduced Bone Marrow

Bone marrow transplantation with transduced stem cells was carried out as follows. We harvested bone marrow from donor C57BL/6 mice, injected intravenously 4 days earlier with 5-fluorouracil (5-FU) to stimulate stem cell division, and exposed it to ecotropic retroviral supernatants. Following infection, we isolated by FACS retrovirally transduced GFP+ cells and injected them into lethally irradiated C57BL/6 recipient mice. We used donor and recipient C57BL/6 mice that are phenotypically distinguishable on the basis of allelic differences at the CD45 locus: donor cells express CD45.2, whereas those of the recipients are CD45.1 [29]. This isolated allelic difference among syngeneic mice does not alter graft tolerance, but enables precise monitoring of the extent of reconstitution with donor-derived cells in transplanted mice [29]. We transplanted a total of 28 mice: 13 with MSCV[FLAG-endostatin/GFP], 5 with MSCV[endostatin/GFP], and 10 with MSCV[GFP] transduced marrow. All mice were kept for a minimum of 3 months post-transplantation and monitored by

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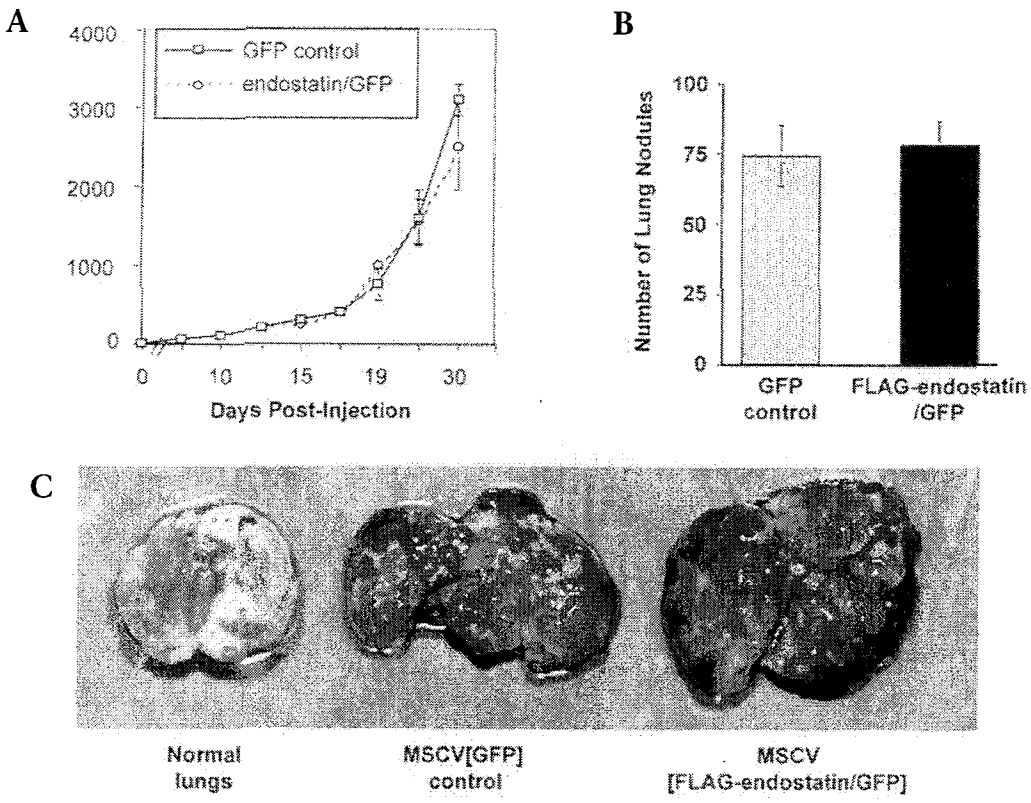

FIG. 3. Quantification of syngenic primary and metastatic tumor growth in transplanted mice with high serum concentrations of endostatin. (A) Growth curves of T241 fibrosarcoma cells injected subcutaneously in syngenic recipients of MSCV[endostatin/GFP] (n = 5) or MSCV[GFP] (n = 4) transduced marrow. Error bars represent standard deviations. (B) Quantification of lung tumor nodules in all mice transplanted with MSCV[FLAG-endostatin/GFP] (n = 13) or MSCV[GFP] (n = 6) transduced marrow, 15 days after intravenous injection of syngenic T241 fibrosarcoma cells. Error bars represent standard deviations. (C) Photographs of lungs from representative mice as described in (B).

quantitative FACS and ELISA analyses of peripheral blood samples. In addition, genomic DNA was isolated from bone marrow and thymus of all mice upon sacrifice for Southern blot analysis. After 3 months post-transplantation, most peripheral blood leukocytes in recipient mice were derived from donor, CD45.2, stem cells and expressed the transferred GFP gene (Fig. 1B). Chromosomal integration of intact provirus in all transplanted mice was confirmed by Southern blot analysis (Fig. 1C).

Sustained, High-Level Expression of Recombinant Endostatin in the Serum of Transplanted Mice

Next, we set out to compare the steady-state serum concentrations of murine endostatin achieved in transplanted mice compared with non-transplanted mice having received 20 mg/kg/day of purified, precipitated recombinant endostatin for 12 consecutive days, a dose that was shown to cause the regression of established primary tumors in mice [4,5]. Serum concentrations were measured with a highly specific polyclonal antibody-based ELISA assay for murine endostatin. As previously observed [30], the average serum levels of recombinant endostatin following the 12-day, high-dose subcutaneous injection regimen were not raised over background levels: 117 ± 16 ng/ml (n = 4) versus 129 ± 16 ng/ml (n = 4), respectively (Fig. 1D). A plausible explanation for the low serum levels achieved by subcutaneous injection of non-soluble endostatin is that the molecules of endostatin, which are slowly released from the depot, rapidly bind to the endothelial cell lining of vessels [28]. In contrast, the production of endostatin by transduced blood and marrow cells of transplanted mice was such that serum endostatin levels rose to 746 ± 197 ng/ml (n = 18; Fig. 1D). No statistical difference was detected between levels achieved with MSCV[FLAG-endostatin/GFP] and MSCV[endostatin/GFP] viruses. Thus, the levels of recombinant endostatin present in the serum of mice transplanted with endostatin transduced marrow was, on average, 7.5-fold greater than the levels detected in the serum of mice following the 12-day injection regimen with 20 mg/kg/day of purified protein (Fig. 1D).

Lack of Inhibition of Neoangiogenesis, Primary Tumor Growth and the Growth of Metastases in Recipients of Endostatin Transduced Bone Marrow

To determine whether inhibition of neoangiogenesis could be detected *in vivo* in transplanted mice that had high steady-state serum levels of endostatin, we carried out a corneal micropocket assay [31]. No inhibition of angiogenesis was observed in mice transplanted with MSCV[endostatin/GFP] transduced marrow (n = 5) compared with control MSCV[GFP] mice (n = 4; Fig. 2). Also, we observed no obvious abnormalities or delay in the healing of tail wounds following peripheral blood isolation in any of the mice throughout the 4-month post-transplantation period.

To investigate whether continuous, high-level, intravascular secretion of murine endostatin would inhibit primary tumor growth in transplanted mice, we injected syngeneic T241 fibrosarcoma cells subcutaneously and measured tumor growth for 30 days. All the mice that were submitted to the corneal micropocket analysis were included in this tumor assay. T241 fibrosarcoma was chosen because it had been reported to be effectively placed in a permanent state of tumor dormancy when tumor-bearing mice were injected subcutaneously with purified, precipitated recombinant endostatin [4,5]. A surprising finding was that no inhibition of tumor growth was observed in recipients of MSCV[endostatin/GFP] (n = 5) transduced marrow as compared with recipients of control MSCV[GFP] transduced marrow (n = 4; Fig. 3A). We next asked whether transplanted mice would be refractory to the development of metastases in an intravenous quantitative metastatic model. We injected recipients of MSCV[FLAG-endostatin/GFP] (n = 13) and MSCV[GFP] (n = 6) transduced marrow with syngeneic T241 fibrosarcoma cells, and 15 days later the lungs were removed and analyzed (Figs. 3B and 3C). No decrease in the number or growth rate of lung tumor nodules was detected (Figs. 3B and 3C).

Although bone marrow engraftment with neo-antigens following lethal irradiation is known to induce a state of tolerance in most instances [32], we analyzed serum samples for the possible presence of neutralizing antibodies. No antibody directed against recombinant endostatin could be detected in the serum of transplanted mice.

Discussion

Here, we have demonstrated that transfer of a retroviral vector encoding a secretable form of murine endostatin into hematopoietic stem cells results in high-level, long-term secretion of the protein in the vasculature of all transplanted mice. Despite this achievement, however, no inhibition in neoangiogenesis or the growth of primary or metastatic tumors was observed. Similiar results were obtained using a NOD/SCID model of human leukemia in the companion paper in this issue [33]. How could these unexpected findings be explained? Serum endostatin concentrations in transplanted mice were 7.5-fold higher than those observed in mice having been submitted to a 12-day regimen of subcutaneous injection of high-dose (20 mg/kg/day) recombinant protein. In our hands, no significant increase in serum concentration could be detected following this regimen of repeated subcutaneous injection of recombinant endostatin. Accordingly, no significant inhibition of tumor growth could be documented ([30] and data not shown). The reasons for this discrepancy with other reports are unknown. The serum levels of endostatin documented in this study were within the range observed in studies using recombinant adenoviral vectors in which an anti-angiogenic and/or anti-tumor effect was reported [23–27]. Thus, it is unlikely that serum concentrations were below therapeutic levels. Furthermore, the integrity of the secreted proteins was verified by western blot analysis and microsequencing, and no neutralizing antibody was detected in the serum of transplanted mice. In addition, the biological activity of recombinant endostatin present in supernatants of transduced cells was verified by its ability to specifically inhibit the proliferation of bovine endothelial cells *in vitro*. Loss of activity by potential degradation of the protein termini is unlikely, as recombinant endostatins lacking N- and/or C-terminal pentapeptide sequences are as active as intact endostatin [34]. It is also unlikely that potential improper folding of the secreted endostatin was to blame, as the most active protocol so far makes use of bacteria-derived recombinant endostatin that precipitates after purification [4]. It is possible that continuous secretion of endostatin does not provide the benefits of intermittent, cycled therapy, for reasons we do not understand. Although much is known with regard to the ability of endostatin to induce endothelial cell apoptosis [35] and its interaction with a variety of extracellular matrix proteins [10] and heparin sulfate [28], much of the molecular details of its mode of action remain unknown. Recombinant endostatins produced in yeast [8,9,12], mammalian cells [10,11,34], or tumor cells following gene transfer [17,18] have shown perceptible activity in mouse tumor models, albeit without evidence of tumor regression. Because the variability in tumor growth following subcutaneous injection of tumor cells is considerable, the resolution of the paradox uncovered by various studies will probably await the results from animal experiments and human clinical trials conducted with large numbers of subjects.

Here, we established that transfer of a retroviral vector encoding a secretable form of a putative antiangiogenic protein into hematopoietic stem cells results in high-level, long-term secretion of the protein in the vasculature of all transplanted mice. This approach should be of substantial value in the identification and assessment of novel angiogenic inhibitors with antitumor properties.

Materials and Methods

Vector construction and cell lines. The mouse procollagen type XVIII α1 cDNA [36] was used as a template to amplify the published sequence of murine endostatin [4] by PCR using two sets of primers encoding the murine IgGκ secretory signal (pSecTag, Invitrogen, Carlsbad, CA), and either did or did not encode the FLAG tag (IBI, New Haven, CT). Integrity of the secretory signal, FLAG tag, and endostatin coding sequence was verified by sequencing both DNA strands. Three vectors were constructed. All vectors were based on the MSCV backbone [37] and contained, 3′ of the endostatin cDNA, the post-transcriptional regulatory element (PRE) from the hepatitis B virus [38] and the IRES element from the encephalomyocarditus virus (Novagen, Madison, WI) fused 5′ to the GFP gene (Clontech, Palo Alto, CA). The amino acid sequence of the secreted forms of endostatin encoded by FLAG-tagged and non-tagged endostatin was NH2-AGDLDTKDDDDKLAHTH and NH2-AAQHTH respectively (the N-terminal residues of murine endostatin are underlined). The amino acid residues 5′ of the non-tagged endostatin sequence represent residues encoded by engineered cloning sites. The third vector, containing all

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of the aforementioned elements but lacking an endostatin cDNA, was constructed as a control. T241 fibrosarcoma cells (a gift from Judah Folkman, Children's Hospital, Boston, MA), 293T cells, and the transient packaging cell line BOSC 23 [39] were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with penicillin/streptomycin (Pen/Strep) and 10% heat inactivated (30 minutes at 55°C) fetal calf serum (Hyclone, Logan, UT). Primary bovine capillary endothelial (BCE) cells were isolated and cultured as described [40].

Preparation of purified recombinant murine endostatin from E. coli. The 552-bp PCR fragment encoding murine endostatin was cloned into the PQE.40 expression vector (Qiagen, Valencia, CA) 3′ of a leader sequence encoding a 6 × His tag. The N-terminal amino acid sequence of the fusion protein encoded by this vector was NH_2-MRGSHHHHHHGSGGKLAHTH (the N-terminal residues of murine endostatin are underlined). This vector was transformed into the *E. coli* strain MP15 (Qiagen, Valencia, CA). Purification under denaturing conditions was performed according to the manufacturer's instructions (Qiagen, Valencia, CA). After elution from Ni^{2+}-NTA beads, the solution was dialyzed against PBS at 4°C for 72 hours using Spectrum spectra/pore membrane MWCO 6000–8000 kDa (Spectrum Laboratories Inc., Rancho Dominguez, CA). Approximately 90% of the purified endostatin precipitated during this step and was collected by centrifugation. The precipitated fraction was resuspended in PBS and its concentration assessed by weighing a desiccated aliquot. Precipitated endostatin was adjusted to a concentration of 3 mg/ml, aliquoted in 300 μl fractions, and used for the *in vivo* experiments.

Generation of recombinant retrovirus stocks. Retroviral construct DNAs were introduced into the transient packaging cell line BOSC 23 [39] using a classical $CaPO_4$ transfection method. Supernatants were collected 48 hours later, filtered through 0.45-μm filters, and either used immediately or frozen at –80°C. Viral titers were determined by exposing 1×10^5 NIH3T3 cells to serial dilutions of filtered virus preparations in the presence of 8 μg/ml protamine sulfate, and assessing the proportion of GFP+ cells by flow cytometry 48 hours later. The presence of replication competent retrovirus (RCR) was assessed by the ability to serially transfer viruses conferring G418 resistance to NIH3T3 cells.

Transduction of mouse bone marrow with recombinant retrovirus and assessment of reconstitution with donor cells. Transduction of bone marrow cells was carried out as described [41]. Briefly, bone marrow cells obtained from the hindlimbs of CD45.2 donor mice (The Jackson Laboratory, Bar Harbor, ME), injected 4 days previously with 150 mg/kg 5-fluorouracil (5-FU), were prestimulated for 48 hours in α-medium supplemented with 15% fetal calf serum (Stem Cell Technologies Inc., Vancouver, Canada), 10 ng/ml human IL6, 6 ng/ml murine IL3, and 100 ng/ml murine stem cell factor. All cytokines were bought from PeproTech (Rocky Hill, NJ). Cells were exposed to filtered virus preparations, supplemented with the above cytokines, on Retronectin (Biowhittaker, East Rutherford, NJ) coated tissue culture dishes for 2 days and GFP+ cells were sorted by FACS 48 hours postinfection. Following selection 5×10^5 to 1.4×10^6 GFP+ bone marrow cells were injected into CD45.1 recipient mice (National Cancer Institute, Bethesda, MD) given 950cGy of whole body irradiation. Blood samples (50 μl) were depleted of erythrocytes by incubation for 10 minutes on ice in the presence of 4 volumes of 1 mol/L NH_4Cl solution and stained with 1 μg/ml phycoerythrin-labeled anti-CD45.2 antibody (Pharmingen, San Diego, CA). Cells were washed with PBS containing 2 μg/ml propidium iodide (PI; Sigma, St. Louis, MO) to distinguish dead cells and concurrently analyzed for GFP and phycoerythrin mediated fluorescence on a FACScan flow cytometer (Becton Dickinson, San Diego, CA).

Southern blot analysis. Genomic DNA was isolated from primary bone marrow and thymus using DNAzol (Invitrogen, Carlsbad, CA). Genomic DNA (10 μg) was digested with *Sac*I, electrophoresed through a 0.8% agarose TAE gel, and transferred to nitrocellulose overnight by capillary action. Membranes were probed with the GFP cDNA labeled with α-^{32}P by random oligo priming. Blots were exposed to Kodak XAR film at –70°C for 1–2 days.

Endothelial proliferation assay. Bovine capillary endothelial (BCE) cells were isolated and cultured as described [40]. Cells growing in gelatinized six-well plates were dispersed in 0.05% trypsin solution and resuspended in medium containing 5% bovine calf serum (BCS). After trypsinization, approximately 1×10^4 cells in 0.5 ml medium were added to each gelatinized well of 24-well plates and incubated at 37°C for 30 minutes. Samples of endostatin were analyzed in triplicate. Endostatin samples were added to each well and after 30 minutes of incubation, FGF2 (Peprotech) was added to a final concentration of 1 ng/ml. After 72 hours, cells were trypsinized and counted with a Coulter counter (Coulter Electronics Ltd., Fullerton, CA).

Mouse corneal micropocket assay. The corneal micropocket assay was performed as described [31]. Briefly, micropockets were created in the corneas of transplanted mice with a cataract knife. A sucrose aluminum sulfate, hydron polymer pellet coated with 80 ng FGF2 was implanted into each pocket and 5 days later corneal neovascularization was examined with a slit lamp biomicroscope. Vessel length and clock hours of circumferential neovascularization were measured as described [31].

Quantification of tumor growth in recipient mice. For primary tumor studies, 1 million syngeneic T241 fibrosarcoma cells were resuspended in 0.1 ml PBS and subcutaneously injected into each bone marrow recipient at the dorsal midline. Tumor volumes were calculated according to the following formula: $\text{width}^2 \times \text{length} \times 0.52$ [4,5]. For metastases assays, 5×10^5 non-transduced T241 fibrosarcoma cells were intravenously injected into each transplanted recipient mouse in a total volume of 0.1 ml PBS.

Quantification of recombinant endostatin in the serum of recipient mice. Quantification of murine endostatin in mouse serum was carried out using the Accucyte ELISA kit according to the manufacturer's instructions (Cytimmune, College Park, MD).

Purification and analysis of secreted recombinant endostatin. Serum-free supernatants from endostatin and control vector transduced 293T cells were harvested and used for purification of endostatin with heparin-Sepharose (Pharmacia, Piscataway, NJ). Approximately 2 ml supernatant was mixed with 200 μl of 20% heparin-Sepharose in TNE buffer and rotated overnight at 4°C. Beads were washed five times with TNE buffer and the bound materials were released by addition of sample buffer containing SDS and DTT, and boiling for 4 minutes. Samples were run on SDS-PAGE gels and transferred to nitrocellulose membrane. Membranes were incubated with a polyclonal rabbit-anti-mouse endostatin antibody (gift of Judah Folkman, Childrens Hospital, Boston, MA) diluted 1:2000 in PBS. Membranes were then incubated with donkey-anti-rabbit IgG HRP-labeled secondary antibody (Amersham, Piscataway, NJ) diluted 1:2000 in PBS. Proteins were visualized using the ECL+ system (Amersham, Piscataway, NJ). To prepare endostatin samples for microsequencing, supernatants from endostatin-transduced 293T cells were loaded onto a heparin-Sepharose 6B column equilibrated with TNE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 100 mM NaCl). The column was washed five times with 10 ml TNE and endostatin was eluted with 10 ml of 0.75 M NaCl in 10 mM Tris and 1 mM EDTA buffer. The sample was dialyzed against water at 4°C overnight, lyophilized, and resuspended in water. Endostatin was further purified by size-fractionation chromatography using Sepharose 12 coupled to FPLC, pre-equilibrated with 20 mM Tris, pH 8.0. Fractions containing endostatin were concentrated and desalted with Centricons (Millipore, 10 kDa molecular weight cutoff). N-terminal microsequencing was performed for both tagged and untagged endostatin by the Massachusetts Institute of Technology Biopolymers Laboratory.

Analysis of serum for the presence of neutralizing antibodies to murine endostatin. We coated 96-well ELISA plates (Co-Star, Acton, MA) with either native recombinant murine endostatin (Accucyte ELISA kit) or FLAG peptide overnight at 4°C. Plates were washed three times with wash buffer (PBS + 0.05% Tween 20) and blocked for 2 hours at room temperature with 0.1% BSA in 0.9% NaCl and 2% sucrose solution. Wells were incubated, for 45 minutes, with dilutions of serum from control, non-transplanted mice, or mice transplanted with MSCV[FLAG/endostatin/GFP] or MSCV[endostatin/GFP] transduced bone marrow. Plates were washed and incubated with goat-anti-mouse IgG [Fc-specific] horseradish peroxidase conjugated polyclonal antibody (Oncogene Research Products, Cambridge, MA) diluted to 1 μg/ml in PBS + 0.2% BSA for 45 minutes at room temperature. After washing, plates were incubated with tetramethylbenzadine liquid substrate (Sigma) for 45 minutes and stopping reagent was added. Optical density was determined using an optical plate reader at 450 nm (Tecan, Durham, NC).

Statistical analyses. All statistical analyses were performed with the nonparametric Mann-Whitney U-test.

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Unfulfilled Promise of Endostatin in a Gene Therapy-Xenotransplant Model of Human Acute Lymphocytic Leukemia

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Retroviral transduction of hematopoietic stem cells (HSCs) offers an attractive strategy for treating malignancies that home to the marrow. This approach should therefore be of interest for evaluating the therapeutic activity of anti-angiogenic agents on hematopoietic malignancies whose growth has been associated with enhanced angiogenesis. A variety of studies have indicated endostatin to be a potent anti-angiogenic agent both *in vitro* and *in vivo*, and a human malignancy that might be sensitive to endostatin is human B-lineage acute lymphoblastic leukemia (B-ALL). The demonstrated ability of human B-ALL cells to engraft the marrow of immunodeficient mice suggested the potential of this system for testing an endostatin delivery strategy using co-transplanted non-obese diabetic–*scid/scid* (NOD/SCID) HSCs engineered to express endostatin. Here we show that, in spite of their mutant *scid* gene, NOD/SCID HSCs can be transduced with an endostatin-encoding retrovirus at efficiencies that result in a several-fold increase in endostatin serum levels in transplanted recipients. However, this did not alter the regrowth of co-transplanted human B-ALL blasts. These findings validate this gene transfer approach for investigating effects of novel therapeutics on primary human malignant cells that engraft NOD/SCID mice and question the utility of native endostatin for controlling human B-ALL *in vivo*.

Key Words: angiogenesis, immunodeficient mice, ALL, gene transfer, gene therapy

INTRODUCTION

Expanding populations of malignant cells depend on the continuous outgrowth of new blood vessels, a process referred to as angiogenesis [1]. Previous studies have demonstrated increased angiogenesis in several malignancies, including those of the hematopoietic system [2–5]. One of the first examples was in pediatric acute lymphocytic leukemia (ALL) [2]. In this study, an increased microvessel density relative to normal healthy controls was seen in the bone marrow (BM) at diagnosis, which then returned to normal after achievement of complete remission. These investigators also found increased levels of a potent angiogenic inducer, basic fibroblast growth factor (bFGF), in the serum and urine of these patients at diagnosis. These findings support the hypothesis that angiogenesis may be an important therapeutic target in several human cancers, including some of hematologic origin. Several reports of potent anti-tumor effects of endostatin (a recently identified peptide inhibitor of angiogenesis) in experimental animal models [6–12] have created much excitement in the potential clinical application of this agent and clinical trials have been initiated [13].

To improve delivery of endostatin *in vivo*, a variety of gene transfer approaches have been evaluated. These include *in vivo* injections into a variety of sites of the endostatin gene itself [14–16], or adenoviral vectors encoding this gene [17–22], or cells previously engineered *ex vivo* to produce endostatin for sustained periods; either directly into the experimental tumor or intravenously into the host animals to convert the vasculature into producers of endostatin [23–26]. Although significant anti-tumor effects have been reported in many of these studies, in others a positive result has been either partial or not detectable, even when very high levels of endostatin have been produced [20,21,27].

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TABLE 1: Frequency of transduced NOD/SCID BM stem cells obtained from variously enriched starting populations and levels of GFP+ blood cells 4 months after injection of unselected cells

Target cells[a]	Cytokines[a]	Exp. No.	% GFP+ cells post-transduction[b]	Starting equivalent cells injected per mouse[c]	GFP+ mice/ total mice	Frequency of transduced stem cells in the transplant[d]	Levels of GFP+ cells in positive mice
Day 4 5-FU	S,3,6	1	4.5	3.2×10^6	2/7	5.8×10^{-5}	5, 11
		2	3.7	5.2×10^6	3/7		0.7,3,7
		3	3.9	6.3×10^6	4/9		0.7,2,21,24
	F,S,11	4	3.8	3.4×10^6	2/7	4.8×10^{-5}	8,10
		5	5.1	3.6×10^6	2/8		7,12
		6	4.2	3.8×10^6	4/12		7,11,13,19
		7	3.7	5.1×10^6	2/6		4,5
		8	5.6	5.5×10^6	4/11		6,6,13,15
		9	4.2	7.1×10^6	3/6		4,5,11
		10	3.6	1.2×10^7	4/6		9,9,10,14
Lin-	F,S,11	11	21	2.3×10^4	2/7	3.9×10^{-4}	7,8
		12	13	5.3×10^4	1/6		11
		13	22	6.5×10^4	2/10		1,13
Sca-1+Lin-SP	F,S,11	14	14	1.6×10^3	0/5	2.6×10^{-3}	-
		15	17	4.0×10^3	1/5		11
		16	16	4.5×10^3	2/6		1,10

[a]Day 4 5-FU marrow cells or FACS purified subsets of normal marrow cells from NOD/SCID mice were prestimulated with the cytokines indicated for 2 days and exposed to VCM for 3 days in the presence of the same cytokines (as described in the Methods) prior to transplantation into sublethally irradiated syngeneic hosts.
[b]This value refers to the percent of cells recovered *in vitro* at the end of the 5 day prestimulation-transduction protocol that were GFP+.
[c]Each mouse was injected with the progeny produced during the 5 day prestimulation-transduction protocol from the number and type of original target cells indicated.
[d]This value refers to the calculated frequency of transduced repopulating stem cells among the cells actually transplanted based on the proportion of recipient mice found to contain some detectable GFP+ cells in the blood (> 5 / 1,000 events analyzed). This calculation was performed using the L-calc software program (StemCell).
S, SF; 3, IL-3; 6, IL-6; 11, IL-11.

These findings emphasize the need to carefully define the spectrum of primary human tumor types that may be usefully treated by endostatin and under what conditions. For hematopoietic malignancies, a genetic approach using transplantable hematopoietic stem cells (HSCs) transduced with a retroviral vector encoding endostatin offers an alternative strategy for increasing local delivery, as this should ensure sustained endostatin production directly at the site of malignant cell growth, that is, within the extravascular BM space. To test this concept, we first defined conditions for achieving adequate levels of retrovirus-mediated gene transfer and expression in the progeny of transplantable HSCs from nonobese diabetic-*scid/scid* (NOD/SCID) mice using a murine stem cell virus (MSCV)-based IRES retroviral vector described in detail in the accompanying study [28]. We then co-transplanted sublethally irradiated NOD/SCID mice simultaneously with NOD/SCID HSCs engineered to express endostatin together with primary human ALL cells, having shown in parallel experiments that primary human ALL cells engraft the BM of NOD/SCID mice at high efficiency, a finding that was recently confirmed independently [29]. Using this approach, we found that the growth of human ALL cells in NOD/SCID mice is unaffected by co-transplanted BM cells able to achieve elevated levels of circulating human endostatin for at least 3 months post-transplant.

Results

Retrovirus-Mediated Gene Transfer to NOD/SCID HSCs

It has been reported that the *scid* mutation causes defective retroviral integration resulting from associated low levels of DNA-dependent protein kinase [30]. This suggested that standard protocols for transducing murine

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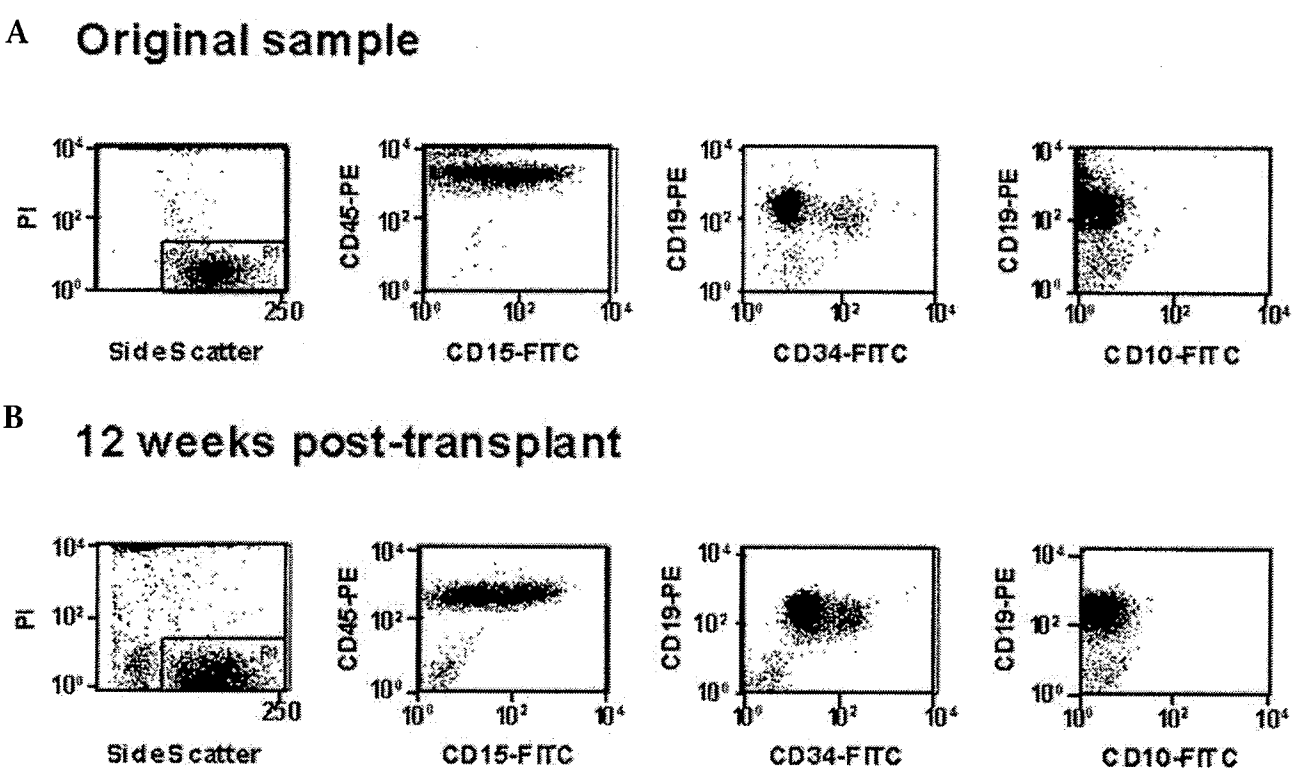

FIG. 1. Human B-ALL cells in engrafted NOD/SCID mice maintain their original surface antigen profile. Data are from a representative patient. (A) Immunophenotype of the cells before injection ($CD45^+$, $CD19^+$, partially $CD15^+$, partially $CD34^+$, $CD10^-$). (B) Immunophenotype of the cells after engraftment of NOD/SCID mice.

HSCs with retroviral vectors might not be effective on NOD/SCID targets. Moreover, as we planned to use sublethally irradiated NOD/SCID mice as hosts, it was additionally important to determine whether useful levels of engraftment with transduced cells could be achieved under these competitive conditions. Recent studies have defined growth factors that optimize normal mouse HSC self-renewal divisions *in vitro* (FLT3 ligand (FL), Steel factor (SF), and interleukin (IL)-11) and the period required for such divisions to be initiated (4 days) [31,32]. This made it possible to design HSC gene transfer protocols that use untreated NOD/SCID mice as BM cell donors rather than mice injected with 5-FU 4 days before, a treatment that reduces the absolute HSC content of the femur by approximately threefold [33]. Table 1 summarizes the pooled results of several experiments in which the same GFP virus (SM10) was used in the different gene transfer protocols compared. These protocols used variously enriched HSC populations obtained from untreated normal adult NOD/SCID BM cells that were maintained *in vitro* for a total of 5 days with FL, SF, and IL-11, as well as BM cells from NOD/SCID mice treated 4 days previously with 5-FU and then cultured also for a total of 5 days, but with SF, IL-3, and IL-6 to allow comparison with a procedure that has been more widely used by other groups. It can be seen that all of the procedures evaluated yielded transplantable NOD/SCID HSCs capable of producing detectable levels of GFP^+ myeloid progeny for at least 4 months in sublethally irradiated syngeneic NOD/SCID recipients. In all groups it can be estimated from limiting dilution analysis principles that there was approximately one transduced HSC in each transplant dose tested because the proportion of positive mice was generally ≤ 30%. Consistent with this estimation was the observation that, on average, ~ 8% of the circulating WBCs in each of the mice that regenerated any detectable GFP^+ cells were GFP^+, regardless of the method of transduction used. We have previously shown that each adult BM HSC will produce, on average, 8% of the regenerated blood cells in recipients transplanted with less than 10 HSCs [32]. Unfortunately, cell losses inherent in obtaining the HSC-enriched suspensions from untreated NOD/SCID mouse BM proved to be much higher than those resulting from the 5-FU treatment (data not shown). Therefore, for practical reasons, we used BM cells from 5-FU-treated donors in subsequent experiments. In addition, given the modest gene transfer efficiencies obtained using the supernatant infection protocol, subsequent experiments also made use of a co-cultivation protocol. As can be seen below, we then obtained slightly higher levels of GFP^+ NOD/SCID blood cells in the transplanted recipients.

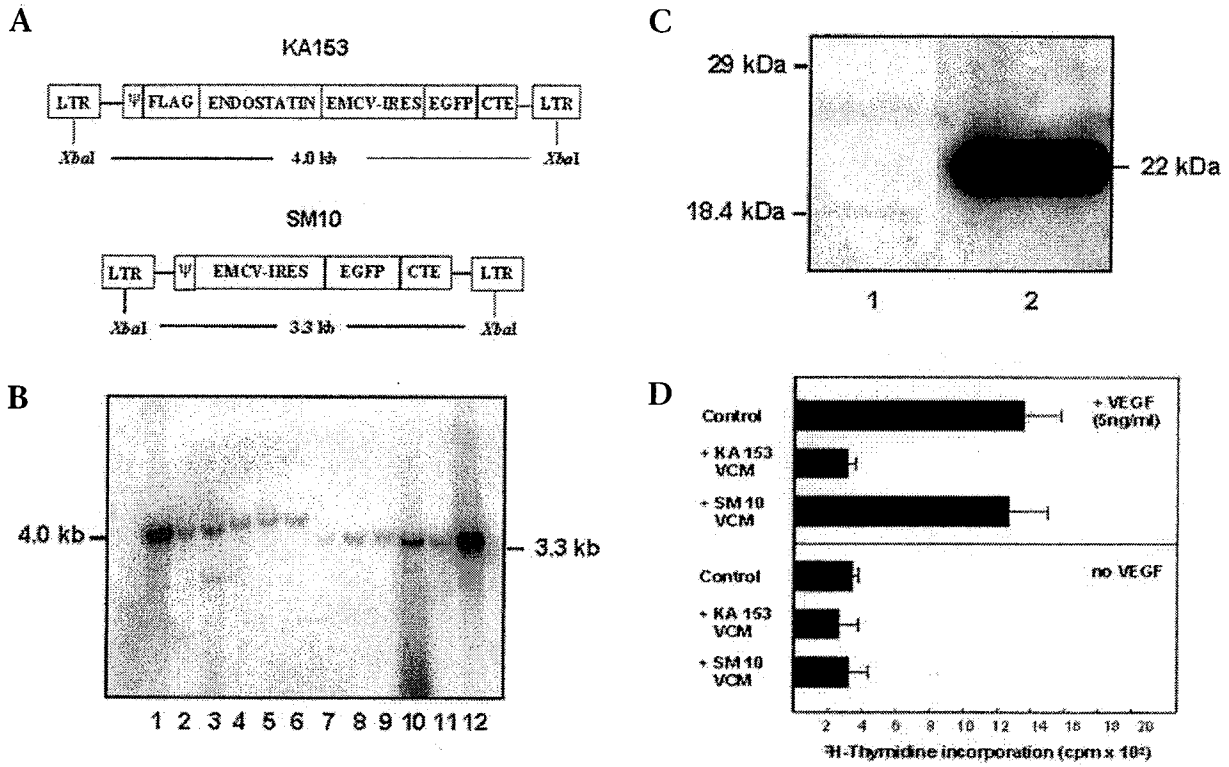

FIG. 2. Structure and characterization of vectors. (A) Structure of the retroviral vectors used in this study. The SM10 MSCV vector incorporates a 515-bp EMCV-IRES fragment, a 700-bp fragment containing the EGFP coding sequence, and a 565-bp fragment containing the constitutive transport element of the hepatitis B virus. The KA153 vector is a derivative of the SM10 vector and also contains the coding sequence of endostatin that was PCR-derived from the C-terminal sequence of murine collagen XVIII. Expected full-length transcripts are shown. MSCV LTR, murine stem cell virus long terminal repeat; ψ, extended packaging signal; EMCV-IRES, internal ribosome entry site of the encephalomyocarditis virus; CTE, constitutive transport element of the hepatitis virus. (B) Detection of intact provirus in FACS-selected (GFP+) transduced NOD/SCID BM cells (lanes 2 and 11) and NIH3T3 cells (lanes 3 and 10), and in the BM of representative NOD/SCID mice transplanted 12 weeks previously with 10^5 GFP+ syngeneic BM cells transduced with either KA153 (lanes 4–6) or SM10 (lanes 7–9). DNA (10 μg) from each tissue sample was digested with *Xba*I, an enzyme that cuts once within each proviral LTR. Shown are the results of a blot probed with a ^{32}P-labeled GFP probe. The positive control represents 50 ng DNA obtained from the original plasmids of KA153 (lane 1) or SM10 (lane 12). Right and left margins, molecular size markers. (C) Detection of endostatin protein in KA153 VCM by immunoprecipitation and western blot. Supernatant of subconfluent KA153 cells (lane 2) and parental GP+E86 control cells (lane 1) was incubated overnight with anti-FLAG M2 beads. Right and left margins, protein size markers. (D) Inhibition of [^{3}H]thymidine-incorporation by HUVEC in culture following the addition of KA153 VCM. HUVEC cells were first plated in medium containing 15% FCS plus 60 μg/mL endothelial cell growth supplement (ECGS) and then for an additional 24 hours in medium containing only 5% FCS (no EGCS). VEGF (5 ng/mL) alone (Ctrl) or with KA153 VCM (KA153) or SM10 VCM (SM10) was then added. After another 24 hours, 1 μCi [^{3}H]thymidine was added and the cultures incubated for a final 24 hours. Starved HUVEC to which VEGF was added served as internal negative controls.

Engraftment of NOD/SCID Mice with Human ALL Cells

We assessed samples from seven patients with ALL (five with B lineage disease, two with T lineage disease) for their ability to grow in sublethally irradiated (350 cGy) NOD/SCID mice after intravenous injection of 10^5, 10^6, and 10^7 cells (three to six mice per cell dose per sample). None of the recipients of the T-ALL cells showed any evidence of human cells for up to 24 weeks post-transplant, at which time the experiments were terminated. Although T-ALL engraftment has been noted [34], 10-fold higher doses of cells were injected. In contrast to our findings with T-ALL, cells from all five B-ALL samples tested did engraft the BM of NOD/SCID mice. The engrafted B-ALL cells were already apparent by 3 weeks post-transplant and at this time they already constituted between 5% and 92% of all the cells present in the BM of the mice. Thereafter, the human B-ALL cells increased in a time- and transplant dose-dependent fashion that was specific for each original B-ALL sample. Figure 1 illustrates the type of patient-specific profile of B-ALL antigen expression that was seen with all five samples at all times post-transplant. From the cell dose response results, frequencies of NOD/SCID repopulating human B-ALL cells in each of the five original B-ALL samples could be determined. These were 0.04 (0.07–0.02), 0.14 (0.28–0.07), 1.4 (2.8–0.7), 95 (1200–1), and $> 10 \times 10^{-4}$ (values shown in brackets indicating the range defined by ± SE). This study thus indicates the relative ease with which human B-ALL cells, even from patients with low-risk disease (absence of cytogenetic abnormalities, $n = 2$),

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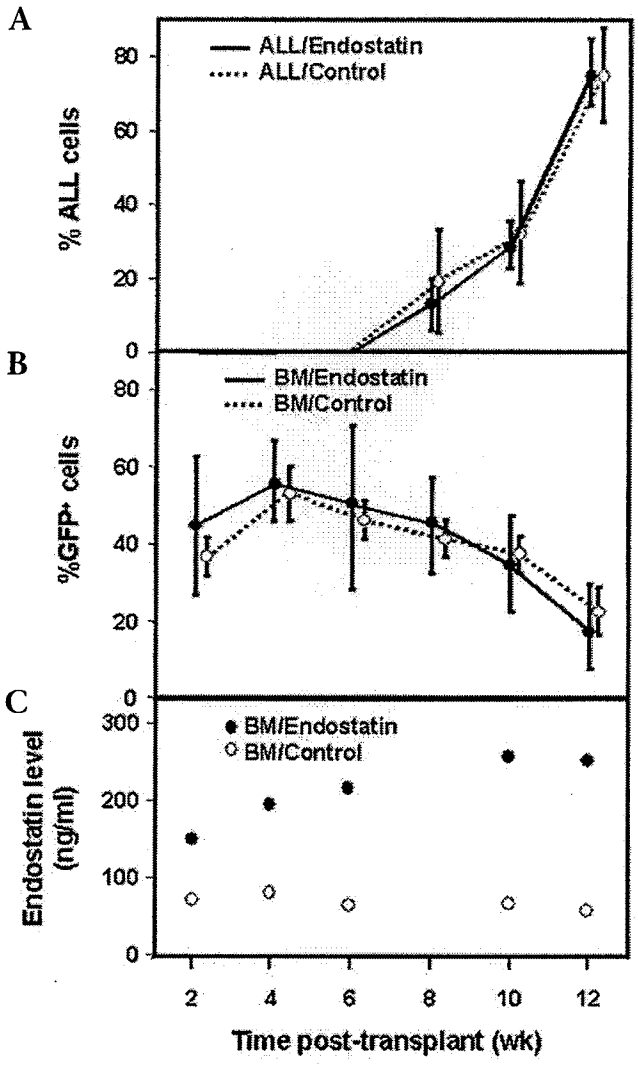

FIG. 3. Kinetics of engraftment of NOD/SCID mice with ALL cells and transduced NOD/SCID BM cells. (A) ALL cell engraftment kinetics in the BM of mice co-transplanted with NOD/SCID BM cells transduced with either the KA153 (endostatin-IRES-GFP) or SM10 (GFP only) vector (eight animals per group). (B) Kinetics of engraftment of the transduced (GFP+) NOD/SCID BM cells in the same mice as in (A) as indicated by their levels of circulating GFP+ WBCs. (C) Endostatin serum levels in the same mice (filled circles show results for recipients of KA153-transduced BM cells, open circles for recipients of SM10-transduced BM cells). Data shown are from mice transplanted with cells from one of four ALL patients.

can engraft irradiated NOD/SCID mice as compared with other immunodeficient strains [35,36], as also reported by others [29].

Unperturbed Engraftment of Human B-ALL Cells in NOD/SCID Mice Co-transplanted with Endostatin-Transduced NOD/SCID BM

To evaluate the ability of endostatin to modulate human B-ALL growth in a vascularized tissue environment, we transplanted irradiated NOD/SCID mice simultaneously with 10^5 cells from four of the five patients' samples with B-ALL together with 2×10^5 transduced, FACS-sorted GFP+ NOD/SCID BM cells. The latter we obtained by co-cultivating cells from 5-FU-treated donors with GP+E86 cells, producing an endostatin IRES-GFP retrovirus (KA153) or a control GFP virus (SM10). These viruses are shown schematically in Fig. 2A. Figure 2B shows the presence of intact provirus in the producer cells and that these producers also generated immunoreactive endostatin protein. Figure 2D shows the results of experiments with human umbilical vein endothelial cells (HUVEC), whose vascular endothelial growth factor (VEGF)-stimulated proliferation could be specifically inhibited by the addition of supernatant from the endostatin vector-producer cells.

Although the pace of human B-ALL cell generation in the BM of the injected mice was again variable according to the particular patient's sample, in none of the four experiments was there any difference between recipients of NOD/SCID BM cells transduced with the endostatin versus the control vector. A representative experiment is shown in Fig. 3. In all groups of transplanted mice, at least 40% of the circulating murine WBCs were GFP+ within 2 weeks post-transplant. However, the numbers of GFP+ WBCs subsequently declined to ~ 20% in each experiment over the ensuing 10 weeks (Fig. 3B). In the serum of the mice that had been co-transplanted with HSCs transduced with the endostatin vector, we observed a consistent, significant ($P < 0.05$), and sustained two- to threefold elevation in endostatin levels (mean ± SE = 180 ± 7 ng/mL, $n = 58$, versus 63 ± 4 ng/mL in recipients of control (SM10)-transduced cells, $n = 10$, and 61 ± 4 ng/mL in untreated NOD/SCID mice, $n = 7$; Fig. 3C). In addition we obtained evidence of endostatin transcripts in the circulating progeny of the transduced cells from RT-PCR analyses of these cells (data not shown).

Discussion

These studies describe the development and application of a unique model for assessing gene therapy approaches to human disease where sustained delivery of a therapeutic product by cells of the blood-forming system would offer advantages in terms of tissue penetration or localization. The use of immunodeficient mice as hosts for the engraftment of human hematopoietic cells, both normal and malignant, is now well established and the particular sensitivity of the irradiated NOD/SCID mouse for this purpose has been widely recognized [37,38]. Here, we have successfully exploited this model to enable a transplant of transduced syngeneic NOD/SCID marrow cells to maximize the *in vivo* production of an anti-angiogenic agent at the site of an expanding population of human B-ALL cells in the absence of any immune effect, thus simulating what

might be expected of a transduced autologous transplant in a clinical setting.

The anticipated barrier to this experimental model, posed by the presence of the *scid* mutation in the NOD/SCID BM cells [30], did not prevent the achievement of useful levels of HSC transduction, although the yield of transduced HSCs was reduced several-fold by comparison with our experience with normal mice. In part, our success in transducing NOD/SCID HSCs may be due to the use of a cytokine cocktail (FL, SF, and IL-II) with more potent HSC-stimulating activity than has historically been used in gene transfer protocols with retroviral vectors. Indeed, we have shown here that this enables useful transduction efficiencies to be achieved with partially purified, but otherwise untreated, normal adult NOD/SCID murine BM HSCs.

Durably elevated circulating levels of endostatin were thus obtained in recipients of HSCs transduced with an endostatin vector and it is probable that much higher levels would have surrounded the human B-ALL cells regenerating in the BM of the co-transplanted mice. Although it is not known whether higher levels of circulating endostatin might have been effective, a concentration of 100 ng/mL has been found sufficient to inhibit endothelial cell proliferation *in vitro* [6], and this was exceeded in the human ALL-engrafted mice studied here. Moreover, the serum levels we documented are similar or higher than those reported by others to be associated with positive effects on tumors of mouse origin [6–8,14]. However, other examples of a lack of anti-tumor activity of even higher levels of circulating soluble endostatin have been recently reported even under conditions where alternative anti-angiogenic agents can be shown to exert such activity [21]. A similar result is documented in the accompanying paper by Pawliuk *et al.* [28]. Thus, our finding that elevated concentrations of endostatin did not affect the rate at which human B-ALL cells expand *in vivo* in the NOD/SCID model from an initially small innoculum (10^5 cells) may be indicative of a broader insensitivity of primary human malignant cells to endostatin therapy. Certainly our results raise questions as to the role of *in vivo* endostatin treatment in human B-ALL where, unfortunately, new approaches are badly needed. On the other hand, the HSC gene transfer model described here should facilitate investigation of other candidate therapeutic agents that can be encoded in retroviral vectors.

Materials and Methods

Mice. NOD/SCID mice [39] were bred and maintained in the animal facility of the British Columbia Cancer Research Centre (Vancouver, BC, Canada) in microisolator cages and provided with autoclaved food and water. Animals were used at 8–10 weeks of age. Irradiated mice were given acidified drinking water supplemented with 100 mg/l ciprofloxicin (Bayer AG, Leverkusen, Germany) for at least 6 weeks.

Human ALL cells. Peripheral blood cells were obtained with informed consent from newly diagnosed or relapsing ALL patients at the time of their routine assessment. The diagnosis of T or B-lineage ALL was based on initial standard morphologic, histochemical, and flow cytometric analyses of these cells as well as cytogenetic analysis of concurrent BM samples. Low-density (< 1.077 g/ml) peripheral blood cells were isolated by centrifugation on Ficoll-Hypaque (Amersham Pharmacia Biotech, Piscataway, NJ) and cryopreserved in Iscove's modified Dulbecco's medium (IMDM, StemCell Technologies, Inc., Vancouver, BC) with 50% fetal calf serum (FCS) and 10% dimethylsulfoxide (Sigma Chemicals, St. Louis, MO). Frozen cells were thawed at 37°C, washed in IMDM with 30% FCS, and cell viability was determined by Trypan blue exclusion.

Retroviral vectors. A control vector (SM10; Fig. 2A) was created from a MSCV vector originally obtained from Robert Hawley (American Red Cross, Rockville, MD) by insertion of a 515-bp *Sal*1/*Nco*1 fragment of an EMCV-intraribosomal entry site (IRES) element (Novagen, Madison, WI), followed by a 700-bp *Nco*1/*Not*1 fragment containing the enhanced green fluorescent protein (EGFP) cDNA (Clontech, Palo Alto, CA) and finally a 565-bp fragment containing a hepatitis B virus post-transcriptional element (HBPRE, from T.S. Benedict Yen, University of California, San Francisco, CA) to promote nuclear export of intronless transcripts [40]. A bicistronic vector encoding murine endostatin (Fig. 2A) was generated as described in the accompanying paper by Pawliuk *et al.* [28]. Viral producer cells were generated using the ecotropic GP + E-86 retroviral packaging cell line [41]. Titers of GFP-encoding virus in medium conditioned by the KA153 and SM10 producers for 36–48 hours (KA153 VCM and SM10 VCM) and then filtered through a 0.45 μm filter (Millipore, Bedford, MA) were 4×10^5 and 10^6, respectively, as assayed on NIH/3T3 target cells. These VCM were shown to be free of helper virus using a NIH/3T3 rescue assay [42]. Southern blot analysis of genomic DNA isolated from transduced NIH/3T3 cells showed the exclusive presence of an intact recombinant provirus upon hybridization with a GFP probe (Fig. 2B). Supernatants of producer cells obtained after a 48-hour incubation period contained murine endostatin (430 ± 44 ng/ml) as determined by a specific ELISA kit (Accucyt Murine Endostain, Cytimmune Sciences Inc., ML) and western blot analysis (Fig. 2C). Further evidence of functional endostatin production by KA153 cells was obtained by incubating starved HUVEC (American Type Culture Collection, Rockville, MD) with 5 ng/ml VEGF (R&D Systems, Minneapolis, MN) with or without KA153 or SM10 VCM and demonstrating a pronounced and specific inhibition of VEGF-stimulated proliferation of the HUVEC in the exclusive presence of KA153 VCM (Fig. 2D).

RT-PCR analyses. Total RNA was extracted with RNAzol reagent (Canadian Life Technologies, Burlington, ON) and reverse transcribed using a random primer pd$(N)_6$ (Amersham Pharmacia Biotech). A 5 μl aliquot of the RT reaction was then subjected to PCR amplification (35 cycles at 94°C for 30 seconds, 62°C for 30 seconds, and 72°C for 1 minute) in 50 μl volumes of 1× PCR buffer (Gibco/BRL, Burlington, ON) containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP (Amersham Pharmacia Biotech), 2.5 units of *Taq* polymerase, and 10 pM of specific primers for KA153 (5'-GGTCTGTGCC-CATCGTCAACCT-3' and 5'-GGTAGCGGCTGAAGCACTGCAC-3') and actin (5'-GTGCGTGACAT-TAAGGAGAA-3' and 5'-GGAGGGGCCGACTCGTCA-3') to give DNA fragments of 1101 bp (KA153) and 470 bp (actin). Aliquots (10 μl) of the amplified PCR products were then electrophoresed in 1% ethidium-containing agarose gel and photographed under UV illumination.

Southern blot analysis. DNA was extracted using DNAzol reagent (Canadian Life Technologies) and then resuspended in 1 × TE (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0). DNA (10 μg) was digested with *Xba*1 at 37°C for 12–16 hours, separated on a 1% agarose gel, transferred to a nylon membrane (Zeta-Probe, Bio-Rad, Hercules, CA), and hybridized overnight at 60°C with GFP and actin probes. These were labeled by incubating the corresponding denatured fragments in the presence of hexamers and the Klenow fragment of polymerase I using a random primer kit (Gibco/BRL) followed by purification on a Sephadex G50 column. Densitometric analysis was performed using a phospho-imager with ImageQua software (Molecular Dynamics, Sunnyvale, CA).

Retroviral gene transfer to NOD/SCID BM cells. For initial studies, HSC-enriched BM cell populations were obtained either by injecting the donors intravenously with 150 mg/kg 5-fluorouracil (5-FU) 4 days previously, or by immunomagnetic removal of cells expressing various lineage markers

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(CD5, CD11b, CD45RA, Ly-6G and the antigen recognized by Ter 119) using a kit from StemCell (to isolate a lin$^-$ fraction), or by fluorescent activated cell sorting (FACS) to isolate Sca-1$^+$lin$^-$ verapamil-sensitive Hoechst 33342^{lo} (SP) cells as described [43]. Each of these three populations was then prestimulated for 2 days with one of two cytokine combinations. These were either 100 ng/ml human FL (Immunex Corp., Seattle, WA) plus 50 ng/ml murine SF (expressed in cDNA-transfected COS cells and purified in the Terry Fox Laboratory) plus 100 ng/ml human IL-11 (Genetics Institute, Cambridge, MA), or 100 ng/ml murine SF plus 6 ng/ml murine IL-3, plus 10 ng/ml human IL-6 (all three of which were made in the Terry Fox Laboratory). These were added to IMDM containing 15% FCS or a serum substitute (BIT, StemCell) plus 10^{-4} M 2-mercaptoethanol and 40 μg/mL low-density lipoproteins (Sigma). The cells were then suspended in VCM supplemented with the same cytokines as used for pre-stimulation plus 5 μg/ml protamine sulfate and placed into Petri dishes that had been precoated with fibronectin (Sigma) and VCM [44]. The next day, the nonadherent cells were removed, resuspended in fresh VCM with the same supplements and returned to the same dishes for another 48 hours. All cells were then harvested, including any adherent cells, using a disposable spatula. For the later studies in which transduced NOD/SCID BM cells were cotransplanted into mice with human ALL cells, day 4 5-FU BM cells were transduced using the same prestimulation protocol (with FL, SF, and IL-11) followed by cocultivation for 3 days with 70% to 80% confluent, irradiated (30 Gy X-rays) producer cells at 10^5 BM cells/ml in DMEM plus 15% FCS, FL, and SF and IL-11 and protamine sulphate at the same concentrations as used previously. BM cells were then collected by repeated rinsing of the feeders. In all cases, GFP$^+$ cells were also assessed and, where indicated, selected by FACS before their injection into mice.

Transplantation and repopulation measurements. NOD/SCID mice were irradiated with 350 cGy from a ^{137}Cs source and injected with GFP$^+$ NOD/SCID BM cells and/or freshly thawed human ALL blasts at the doses indicated. Four months post-transplant, tail vein peripheral blood samples were assessed for the presence of GFP$^+$ leukocytes (≥ 2 × 10^4 blood cells analyzed/mouse) and the proportion of negative mice in any group determined using 0.5% GFP$^+$ myeloid blood cells as the criterion to distinguish positive and negative mice. Note that only myeloid cells could be evaluated because NOD/SCID HSCs do not make lymphoid progeny [39]. The frequencies of repopulating cells in the original GFP$^+$ cell suspension transplanted were then calculated using Poisson statistics and the method of maximum likelihood available in the L-calc software (StemCell). Human ALL cell engraftment was also assessed by analyzing ≥ 2 × 10^4 WBCs obtained from tail vein peripheral blood samples and/or BM aspirates. For the purposes of calculating NOD/SCID leukemia-initiating cell frequencies in the input inoculum, the presence of ≥ 1% of cells expressing human CD19 (using appropriate isotype controls) was used as the cut-off for defining mice as positive or negative. In addition some mice were analyzed for other markers characteristic of the injected human ALL population.

Western blot analysis. Supernatant of subconfluent KA153 cells and parental GP+E86 control cells was incubated overnight with anti-FLAG M2 beads (1:50 dilution) at 4°C. The beads were then washed 3× with DMEM and bound proteins subjected to 12% SDS-PAGE and blotted. The membrane was then pre-blocked with Blotto solution (PBS, 0.05% Tween-20, 5% low fat milk) for 1 hour, incubated with anti-FLAG polyclonal antibodies (Santa Cruz Biotechnology) in Blotto solution for 2 hours, washed six times with PBS/0.05% Tween-20, incubated with horseradish peroxidase-coupled goat anti-rabbit immunoglobin (Jackson Immunoresearch) in Blotto solution for 1 hour, washed six times with PBS/0.05% Tween-20 and developed with an enhanced chemiluminescence kit (ECL, Amersham Pharmacia Biotech).

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Endostatin: Preclinical Development as an Anticancer Agent

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Abstract: Advances in angiogenesis research have shed a new light on the growth and metastasic spread of solid tumors, allowing to define new paradigms for cancer treatment. These studies have highlighted the dramatic therapeutic potential of natural inhibitors of angiogenesis, which were found capable of maintaining tumors in a state of dormancy. One of the most promising of these recently described natural inhibitors of angiogenesis is endostatin, a C-terminal fragment of collagen XVIII. *In-vitro*, endostatin strongly inhibits endothelial cell proliferation and migration. Initial *in-vivo* studies were impressive, recombinant endostatin was shown to induce regression and prevent the growth of experimental tumors in mice. Several studies by independent teams were published thereafter; they either described different forms of the recombinant protein, or developed gene therapy approaches. Most groups have shown perceptible activity in mouse tumor models, albeit without evidence of tumor regression. More recent studies have failed to show any significant antitumor activity. The resolution of these paradoxes is fundamental for obtaining a better view of the therapeutic potential of endostatin. This may require a better understanding of the mechanism of action of endostatin at the molecular level, which remains largely unknown.

1. INTRODUCTION

Angiogenesis refers to the formation of new capillaries from pre-existing vessels. This process plays an essential role in development and reproduction, as well as in wound healing. The induction of angiogenesis is a complex and precise physiological process: it is restricted to limited zones and limited time-periods, but otherwise totally inhibited. On the pathologic side, angiogenesis is a major component of diseases such as arthritis, diabetic retinopathy or cancer [1].

Several lines of evidence demonstrate that angiogenesis plays a crucial role in cancer progression [2]. A classical original experiment showed that an experimental tumor implanted in the rabbit cornea was not able to grow at an exponential rate unless it induced the development of new vascular networks capable of bringing blood to the tumor cells [3]. This was later confirmed on other animals and these model are actually widely used for angiogenesis research [4,5]. Ten years ago, the main molecules involved in tumor growth and invasion were characterized; antibodies directed against these angiogenic factors inhibit tumor growth experimentally *in vivo*, whereas they have no anti-tumor activity *in vitro*, thus confirming the prominent role of angiogenesis in the tumorigenic process [6,7]. Strong clinical arguments favor the importance of angiogenesis in human cancer. Particularly, a significant, independent correlation has been shown between tumor vascularization and prognosis for almost all solid tumors and even for leukemia [8-11].

Physiologically, angiogenesis is a complex and varied process involving various cell types such as endothelial cells, macrophages and mast cells, controlled by a number of angiogenic and anti-angiogenic factors [12]. Evidence increasingly suggests that tumor cells induce angiogenesis in the tumor stroma either through the secretion of angiogenic factors, or the recruitment of normal cells that will secondarily promote angiogenesis [13]. Once they have been activated, endothelial cells start dividing at the same accelerated pace as bone marrow cells [1]. The surface of endothelial cells expresses specific adhesion molecules that are essential to their growth and development, such as integrin $\alpha v\beta 3$ [14]; it also over-expresses growth factor receptors such as Flk-1, the major receptor for *Vascular Endothelial Growth Factor* (VEGF). Endothelial cells migrate in the tumor, organizing into tubes, which finally coalesce into loops, thus causing abnormal vascular remodeling of leaky neo-vessels. Continual angiogenic factor supply is required until maturation [15-17]. The angiogenesis process further depends on proteinases (urokinase, matrix metalloproteinases [MMP]) that target the basement membrane and extracellular matrix [15,18].

Two angiogenic factors seem to have a prominent importance in cancer development: the *Basic Fibroblast Growth Factor* (bFGF), and the VEGF [19]. On the contrary, there seem to be a number of anti-angiogenic factors (reviewed in [20]). Some of them, like interferon-alfa (IFN-α) and interleukin-18, are part of the cytokine family [21,22], which may reflect a possible interdependence between the immune and the vascular systems. More surprisingly, some of the most important angiogenesis inhibitors described to date are cryptic parts of larger proteins that do not have any anti-angiogenic properties in themselves. Such cryptic proteins can be found in Platelet-Factor-4 (PF-4), thrombospondin-1, prolactin, plasminogen, and collagen XVIII [23-26]. Of these, angiostatin (an internal part of plasminogen) and endostatin (a C-terminal

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fragment of collagen XVIII) are among the most potent angiogenesis inhibitors *in vitro* [26,27]. Cryptic angiogenesis inhibitors are not produced directly by any cell type, but they are released after the proteolysis of their precursor. Angiogenesis requires the disruption of extra-cellular matrices; several proteinases are secreted in the tumor stroma during this process. Some of these proteinases are able to release the cryptic inhibitor from its parent protein [28,29]. In this regard, one might consider the production of angiogenesis inhibitors by proteinases as a negative feedback during the angiogenesis process [20,30].

Angiogenesis has been identified as a central pathogenic step in the process of tumor growth, invasion, and metastasis, suggesting the existence of a compulsory route leading tumor cells from the microscopic pre-angiogenic phenotype (dormant tumor) to the macroscopic angiogenic phenotype associated with exponential tumor growth. This phenomenon has been described as the "angiogenic switch". These complex processes involve multiple steps and pathways placed under the dual local control of angiogenic stimulators/inhibitors [1,12]. The theory that has been developed is that of existence of a possible balance between angiogenesis inhibitors and promoters. Angiogenesis would then be caused by an over production of pro-angiogenic factors, or an inhibition of anti-angiogenic factors, both being most likely interdependent [12,31,32]. Furthermore, J. Folkman proposes that the clinical presentation of metastatic disease is dependent on the capacity of metastases to produce their own vasculature. It is suggested that late metastatic relapses are due to dormant pre-existing metastatic cells switching to the angiogenic phenotype. Although in state of active division, those cells are first unable to produce tumors because of their low vascularization, with high hypoxia-induced apoptotic rates counterbalancing cell proliferation. The cells then accumulate genetic mutations and finally acquire an angiogenic phenotype, triggering clinical relapse [32,33].

Controlling angiogenesis is a promising target of cancer therapy. Experimental data described above suggest that treatments reducing the growth of tumor vessels, or molecules preventing cells from switching to the angiogenic phenotype may have potent clinical applications. Moreover, the efficacy of current anticancer treatments is rather low, possibly due to the frequency of acquired genetic mutations in malignant cells and the resulting heterogeneity of their phenotype. Anti-angiogenic drugs target normal cells recruited by the tumor, thus preventing them to evade treatment through acquired genetic mutations [34,35]. Finally, angiogenesis is a minor process in adults. It is mostly implicated in the female reproductive system and during wound healing. Its specific inhibition for the treatment of metastatic cancer is expected to induce acceptable secondary effects [17].

Based on the current knowledge of the mechanisms implicated in tumor angiogenesis, many molecules capable of interacting with angiogenic factors, such as bFGF or VEGF, were developed [6,36-38]. However, due to the multiplicity of angiogenic molecules, a treatment that would block only one endothelial cell activation pathway might prove inefficient in certain tumors where it can easily be "circumvented" by the overexpression of another angiogenic molecule [39]. A therapeutic approach involving direct endothelial cell inhibition should have higher long-term efficacy on a variety of tumors [40].

2. ENDOSTATIN

Endostatin was first isolated from the supernatant of a murine hemangioendothelioma cell line [26]. Sequencing of the protein demonstrated that endostatin is a 20 kDa C-terminal fragment of collagen XVIII, Fig. (**1**). Collagen XVIII, which was first described in 1994, is structurally related to collagen XV and present in multiple organs, with the highest levels in liver, lung and kidney [41]. It was later shown to be mainly localized in the basement membrane zones of the vessels [42], particularly in newly formed, tumor-associated blood vessels [43]. Endostatin is contained within the C-terminal NC1 globular domain of collagen XVIII, which assembles non-covalently into a trimeric structure through an association domain [44]. The NC1 domain of collagen XVIII exhibits striking evolutionary conservation in vertebrates and seems important for regulating endothelial and non endothelial cell mobility in the extra-cellular matrix (ECM) [30]. A proteolytic sensitive hinge region connects the association domain to endostatin, Fig. (**1**). This hinge region is sensitive to cleavage by several proteinases, including Cathepsin L and MMPs, which can release the soluble form of endostatin [45,46]. Serum concentrations of endostatin up to 120-300 ng/ml have been reported in mice and in healthy human donors [44,47]. In cancer patients, several recent publications have shown that higher serum endostatin levels are associated with more aggressive tumors [48-50]. Our team has found a similar pattern in metastatic breast cancer patients, although the

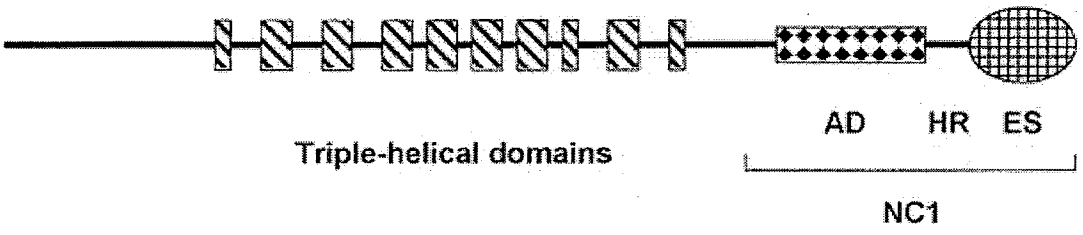

Fig. (1). Shematic structure of collagen XVIII, a member of the multiplexin subfamily of collagenous proteins. Endostatin is located at the C-termini of the C-terminal domain NC1. AS: Association domain; H: Hinge region; ES: Endostatin.

prognostic impact of high serum endostatin in this population is not independent of the classical prognostic factors (T. Bachelot, unpublished results).

The three-dimensional structure of endostatin revealed a complex globular domain composed predominantly of a β-sheet and loops and containing two disulfide bounds [51]. Endostatin has a high affinity for heparin through arginine-rich clusters exposed on the surface of the molecule, and may be associated to a zinc atom through a zinc binding site which closely resembles the structural zinc site of MMP [44,51-53].

2.1. *In-vitro* Activity

Endostatin was initially shown to specifically inhibit endothelial proliferation *in vitro* in a dose-dependent manner at a half-maximal dose ranging from 250 ng/ml to 8 μg/ml, but it has no antimitotic activity on established malignant cell lines [26,47,54]. Subsequent studies showed that endostatin is also an inhibitor of endothelial cell migration and is able to suppress the angiogenic response mediated by VEGF and bFGF in the chorioallantoic membrane assay (CAM assay) [52,55,56]. Endostatin *in-vitro* activity has been subjected to debate since some studies have failed to reproduce the inhibition of proliferation that had originally been described, while showing strong suppression of endothelial cell migration, using as little as 0.1 ng/ml of endostatin [57,58]. Those discrepancies may be related to differences in the endothelial cell assay, the most reliable proliferation inhibition results being obtained with bovine capillary endothelial cells (BCE) and human umbilical vein endothelial cells (HUVEC) [26,47,55,59].

2.2. Mechanism of Action

Numerous studies investigating the potential mechanism of action of endostatin have been published. It was initially hypothesized that zinc binding was necessary for endostatin action, most likely by protecting endostatin from degradation and inducing the specific conformation required for its activation [60]. However, those results were further disproved by two independent teams [52,57].

Soon after the three-dimensional structure of endostatin showing heparin-binding sites was published, another paper reported strong evidence that the action of endostatin was linked to its high affinity to heparan-sulfate; endostatin thereby interacted with crucial growth factors such as bFGF [52]. Endostatin heparin-binding sites might also favor its direct interaction with endothelial cells, and cell-surface glypicans have been shown to serve as low-affinity receptors for endostatin via their heparan-sulfate glycosaminoglycans [61]. Nevertheless, those results were further challenged by another team who demonstrated that endostatin binding to blood vessels is not heparan sulfate dependent, and that bFGF and heparin do not compete for endostatin binding [62].

Another potential effect of endostatin is the induction of endothelial cells apoptosis, as described by two independent teams [56,63]. In both experiments, endostatin-induced apoptosis was particularly evident when endothelial cells were stimulated with bFGF. In the Dhanabal study, apoptosis was induced in 20 to 30% of endothelial cells using 10 μg/ml of endostatin [63], whereas in the Dixelius study only 2 to 4% of the cells were apoptotic when treated with 1 μg/ml of endostatin. In this study, it was reported that endostatin promotes endothelial cell apoptosis by inducing the phosphorylation of the Shb adaptor protein, as was previously shown for angiostatin [56,64]. This effect is dependent on the heparin-binding site of endostatin and not present in the absence of bFGF [56].

Thirdly, endostatin might prevent angiogenesis by interacting with important components of the extra-cellular matrix, such as adhesion molecules and proteolytic pathways. Several studies have provided compelling data in this respect. Y.M. Kim and colleagues first showed that endostatin acts as an MMP inhibitor when used at a concentration of 1 μg/ml [65]. The following year, it was reported that endostatin down-regulates the levels of secreted uPA and PAI-1 by removing uPAR-associated uPA from focal adhesion, thus inducing the disassembly of focal adhesion complexes and the disruption of actin stress fibers in the cytoskeleton [66]. However, by contrast to the former, this study failed to show any action of endostatin on MMP [66]. A recent paper from J. Dixelius similarly reported that endostatin blocks the formation of actin stress fibers and focal adhesions in endothelial cells co-treated with bFGF [67]. In both papers, this *in-vitro* effect is observed with endostatin concentrations ranging from 600 ng/ml to 1 μg/ml [66,67]. It has been shown that endostatin interacts with $\alpha_5\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on the surface of HUVEC cells and that its soluble form functions as an antagonist of integrins, therefore inhibiting endothelial cell migration [68]. This interaction could explain that endostatin has been found to interfere with bFGF rather than with VEGF-induced cell migration and angiogenesis, since $\alpha_5\beta_1$ and $\alpha_v\beta_3$ are known to be more implicated in the former than in the later [52,68,69]. Nevertheless, some authors have reported the opposite, i.e. a better efficacy of endostatin on VEGF induced endothelial cell migration [57]. Finally, Kuo and colleague have shown that the trimerization of the NC1 domain of collagen XVIII positively regulates ECM-dependent mobility of endothelial cells and that this effects is antagonized by unbound monomeric endostatin, which may act as an autoregulatory feedback loop [30].

At the molecular level, M. Shichiri has shown that endostatin down-regulates a variety of genes in growing endothelial cells, including immediate early genes such as c-*myc*, cell-cycle related genes and genes regulating apoptosis inhibitors [58]. Interestingly, his team has transiently overexpressed c-*myc* in an endothelial cell line, with the consequence of abrogating the migratory inhibition effect of endostatin [58]. Another team has shown that endostatin specifically interferes with eNOS phosphorylation in endothelial cell, and therefore significantly reduce VEGF-induced NO-release [70]. More recently, Hanai et al. have reported that endostatin inhibits endothelial cell migration and proliferation by regulating β-catenin stability via a novel GSK3-independent mechanism [71].

2.3. Preclinical Development

The preclinical development of endostatin was started as soon as the protein was described [26]. The seminal paper from M. O'Reilly describes a strong activity of recombinant endostatin on several murine tumor models (Lewis Lung Carcinoma [LLC], T241 fibrosarcoma, B16F10 melanoma, EMOA Hemangioendothelioma, all on C57B16/J mice). Those experimental tumors were treated with 20 mg/kg/day of recombinant endostatin after the tumors had reached 200 mm^3. This treatment resulted in tumor regression in all models. Small residual tumors were described as avascular dormant tumors, i.e. highly proliferative and highly apoptotic [26,33]. The authors described a dose-response effect, with 53%, 97% and 99% inhibition of tumor growth in mice treated with 2.5, 10 and 20 mg/kg, respectively. Discontinuation of endostatin therapy lead to tumor recurrence at the primary site within 5-14 days [26]. A following paper published in *Nature* described cycled endostatin therapy in the same mice [72]. For this experiment, endostatin treatment was stopped when tumor regressed, then resumed when tumor had re-grown to a volume of 300 mm^3, then all over again. It was shown that tumors repeatedly regressed under each treatment cycle. After 6 cycles of treatment for LLC, 4 cycles for T241 and 2 for B16F10, all tumors remained dormant despite discontinuation of endostatin. After 185 days, the mice were sacrificed and it was shown that the remaining dormant tumors had a volume of less that 2 mm^3 [72].

Those experiments raised high expectations in the scientific community and in the public [34,73,74]. Numerous teams, including ours, then began working with endostatin, either to explore the biological process underlying its activity or to develop alternative treatments for common cancers.

Two preclinical approaches were explored and are reviewed below: the first one used different forms of recombinant endostatin produced *in vitro*, and tested different doses and different routes of administration on various preclinical models. The second approach used gene therapy techniques, which offered several advantages: it allowed for a sustained, *in-vivo* production of endostatin and therefore bypassed the needs for complex *in-vitro* production and purification procedures as well as repeated injections [40,75].

2.3.1. Recombinant Protein

As of April 2002, 13 additional preclinical studies using parenteral administration of recombinant endostatin, either alone or in combination with other angiogenesis inhibitor, have been referenced in PubMed. Table **1** lists those studies along with the two original ones [26,72].

One of the first challenges facing the investigators in this new field, was the production of large amounts of recombinant endostatin suitable for the preclinical studies. Original studies by O'Reilly and Boehm used recombinant, N-terminal His-tagged murine endostatin produced in *E. coli*,

Table 1. Preclinical Evaluation of Recombinant Endostatin: Parenteral Administration

Authors	Pub. Date	Protein preparation	Animal model		% Growth Inhibition Treated vs. control	Reference
			Tumors	Mice		
O'Reilly *et al.*	1997	M, *E. coli*, precipitated	LLC, T241, B16F10, EOMA	C57BL/6	Regression	26
Boem *et al.*	1997	M, *E. coli*, precipitated	LLC, T241, B16F10	C57BL/6	Regression	72
Boem *et al.*	1998	M, *E. coli*, precipitated	LLC	C57BL/6	Regression	60
Dhanabal *et al.*	1999	M, Yeast, soluble	HRCC 786-O	Nude	66%	55
Berger *et al.*	1999	M, Ig Fc-endo fusion	pancreatic primary	RIPI-Tag2	5% (NS)	80
Yamaguchi *et al.*	1999	M, 293 Cells, soluble	HRCC RC-9	Nude	59%	57
Sim *et al.*	1999	H, soluble zinc disrupted	LLC, B16-BL6	C57BL/6	85% and 82%	83
Yokoyama *et al.*	2000	M, Yeast, soluble	H ovarian MA148	Nude	5% (NS)	79
Perletti *et al.*	2000	R, *E. coli*, precipitated	Rat mammary prim. tumors	Sprag D. Rat	93%	76
Huang *et al.*	2001	M, *E. coli*, soluble	LLC	C57BL/6	94%	78
Boehle *et al.*	2001	H, *E. coli*, precipitated	KNS-62, Colo-699	SCID	58% (NS) and 67%	77
Jouanneau *et al.*	2001	M, *E. coli*, precipitated	H neuroblastoma SKNAS	Nude	56% (NS)	47
Kuroiwa *et al.*	2001	H, soluble (commercial)	H neuroblastoma TNB9	Nude	46% (NS)	81
Kisker *et al.*	2001	H, soluble zinc disrupted	BxPc-3, HT1080, LLC	SCID, C57BL/6	91%, 81%, 72%	84
Iversen *et al.*	2002	H, soluble (commercial)	HJMML, RAML	SCID, Rats	78%, 46%	82

M: murine; H: human, NS: non significant

which precipitated during purification [26]. Precipitated endostatin was aliquoted and used directly for sub-cutaneous injection. O'Reilly and colleagues have proposed that the injected, non-refolded endostatin protein acts as a subcutaneous depot that results in slow protein release over a 24-48 hr period [26]. This particular administration method was later used to show the importance of the zinc-binding site of endostatin [60]. In this paper, even if soluble endostatin was produced in yeast for *in-vitro* experiments, precipitated fractions from *E. coli* were used in the LLC preclinical model. Results were similar to those of the original study: wild endostatin induced tumor regression while mutants on the putative zinc-binding site showed reduced activity [60]. Three other studies used rat, mouse and human precipitated recombinant endostatin, respectively. Preletti and colleagues showed an important growth inhibition of carcinogen-induced rat primary mammary tumors [76]. Boehle and colleagues, using human precipitated endostatin, showed a non-significant growth inhibition of a human lung cancer cell line, and a significant 67% growth inhibition of a colorectal cell line, both in SCID mice [77]. Our team tested recombinant murine endostatin on a human neuroblastoma xenograft model and observed a non-significant growth reduction of 56% [47].

Other investigators used different soluble forms of endostatin. Huang and colleagues developed a protocol to produce soluble murine endostatin in *E. coli*. Using the LLC pre-clinical model, they showed an equivalent activity of this soluble protein as compared with the precipitated form (both used at 40 mg/kg/day) [78]. The team led by V. Sukhatme produced a soluble form of N-terminal His-tagged murine endostatin in yeast that was tested on a renal cell carcinoma xenograft model in nude mice [55]. Intraperitoneal injections of 10 mg/kg/day did not result in tumor regression per se but allowed for a strong growth inhibition, with a 2.5-fold decrease in tumor volume on the fifth day in comparison to the control. The authors used precipitated recombinant endostatin from *E. coli* as a control and reported equivalent activities of the soluble and the precipitated forms [55]. By contrast, a subsequent experiment using this same yeast-produced, murine endostatin on a preclinical model of human ovarian cancer in nude mice showed poor efficacy, with only a non-significant 5% reduction in tumor growth by day 42. Better results were obtained by combining endostatin and angiostatin, with a potential synergistic effect [79]. One study made use of soluble recombinant endostatin produced in 293 cells on a human renal cell carcinoma xenograft model in nude mice. A significant growth reduction was noted in the treated group for doses as low as 10 μg/kg/day, which is 1,000-fold lower than the doses given to mice in the other studies! Surprisingly, doses over 50 μg/kg/day showed less activity [57]. In one study [80], an immunoglobulin G Fc fragment/Endostatin fusion protein (mFc-mEndostatin) was utilized. In the supplementary material section (www.sciencemag.org/feature/data/990055.shl), the authors reported an unpublished observation from O'Reilly and Javaherian, describing a very high efficacy of this fusion protein in the LLC model. By contrast, the mFc-mEndostatin did not show any efficacy by itself in the RIPI-Tag2 transgenic mouse model when spontaneous pancreatic islet cell tumors were treated after they had reached a mean tumor burden of 77 mm^3 [80]. On the other hand, endostatin was able to reduce the growth of those tumors if the treatment was started when they were only 4 mm^3 and it was shown to reduce the incidence of the angiogenic switch of those spontaneous tumors by 61 % when given "preventively" [80]. This study showed a synergic effect of angiostatin and endostatin on large tumors, as described in the ovarian cancer model [79,80]. Three studies made use of human recombinant soluble endostatin. This protein showed a moderate effect on a human neuroblastoma xenograft model, with a growth inhibition of 46% that was significant only during the first 6 days of treatment [81]. When used on preclinical leukemia models, it allowed a significant reduction of tumor burden [82]. A zinc ligand-disrupted version of this protein was developed for clinical trial and showed equivalent efficacy in preclinical models over the intact protein [83]. In a recent study from Judha Folkman's laboratory, it was shown that zinc ligand-disrupted human endostatin might be more effective when administered as a continuous perfusion [84].

In addition, a report showed that murine soluble endostatin from yeast delayed the onset of adenocarcinoma formation in a transgenic mouse model of spontaneous mammary adenocarcinoma by inhibiting the angiogenic switch [54,85], and short courses of human endostatin were shown to be able to stabilize lymphoma in mice after chemotherapy [86].

2.3.2. Gene Therapy Approach

Twenty studies using some form of gene therapy for *in-vivo* endostatin delivery in cancer preclinical models have been published to date (Table **2**). Five have assessed the tumorgenicity of cell lines engineered *in vitro* to express an endostatin-encoding cDNA. Three of these made use of stable *in-vitro* transfection with plasmid expression vectors [87-89], while 2 made use of retroviral vectors [90,91]. Results showed a significant reduction of tumor growth rates for cell lines expressing endostatin, as compared with a control cell line transduced with the naked vector, which resulted in a significant prolongation of survival. Nevertheless, most endostatin-expressing cell lines were able to grow in the animal and the effect was quite different from one cell line to another. [90,91]. In our own experiments, different retroviral expression vectors were used for transducing cell lines with cDNA encoding a secretable form of endostatin. Those vectors allowed for the expression and secretion of high levels of biologically active endostatin [92,93]. Cell lines transduced and selected for transgene expression were T241 fibrosarcoma, B16F16 melanoma, SAF sarcoma, SKNAS neuroblastoma and TSA breast carcinoma. Implantation into syngeneic mice (or nude mice, for SKNAS), failed to cause any growth retardation of those endostatin-producing tumors as compared with the naked vector-transduced control (R. Pawliuk and T. Bachelot, unpublished results).

Similarly conflicting results were obtained with adenovirus-mediated endostatin gene transfer. Using intratumoral injections of adenoviral vectors delivering endostatin-encoding cDNA, some teams observed a reduction of tumor growth while other failed to observe any effect [94-96]. When those adenoviral vectors were injected

Table 2. Preclinical Evaluation of Recombinant Endostatin: Gene Therapy Approaches

Authors	Pub. Date	Protein	Vector	Delivery	Animal model		% Growth Inhibition Treated vs. control	Ref.
					Tumors	Mice		
Yoon *et al.*	1999	Murine	Plasmid	*In vitro*, stable transfectant	RenCa, SW620	BALB/c, Nude	75%	87
Blezinger *et al.*	1999	Murine	Plasmid	*In vivo*, intramuscular	RenCa, LLC	BALB/c, C57BL/6	42% (Ren), 37% (LLC)	105
Chen *et al.*	1999	Murine	Plasmid/ liposome	*In vivo*, IT or IV	MDA-MB-435	Nude	40% (IT), 37% (IV)	101
Feldman *et al.*	2000	Murine	Adenovirus	*In vivo*, IV	MC38	Nude	40%	98
Chen *et al.*	2000	Murine	Adenovirus	*In vivo*, IV	H colon (prevention)	Nude	Double median survival	99
Sauter *et al.*	2000	Murine	Adenovirus	*In vivo*, IV	JC Breast, LLC	Nude	60% (JC), 78% (LLC)	59
Read *et al.*	2001	Human	Encapsulated cells	*In vivo*, co-injection	BT4C Glioma	BD-IX rats	Double median survival	103
Joki *et al.*	2001	Human	Encapsulated cells	*In vivo*, SC	U87MG Glioma	Nude	62%	104
Scappaticci *et al.*	2001	Murine	Retrovirus	*In vitro* transduction	B16F10, L1210	C57BL/6, BALB/c	50% (B16), Not active (L1210)	90
Jin *et al.*	2001	Murine	Adenovirus	*In vivo*, IT or IV	MidT2, MDA-MB-435	FVB, SCID	67% (MidT2), NS (MDA)	95
Kuo *et al.*	2001	Murine	Adenovirus	*In vivo*, IV	LLC, T241, BxPc3	C57BL/6, SCID	27% (LLC), 0-6%, NS (T241, BxPc3)	97
Hampl *et al.*	2001	Murine	Adenovirus	*In vivo*, IP	TA3, ES-2, SCOV3	Nude	Prolongation of survival	94
Szary *et al.*	2001	Murine	Plasmid	*In vitro* and *in vivo*, IT	RenCa	BALB/c	65% (*in vitro*), 90% (*in vivo*)	88
Sacco *et al.*	2001	Human	Plasmid/liposome	*In vivo*, IP	Spontaneous breast tumor	MMTV-*neu*	50%	106
Régulier *et al.*	2001	Murine	Adenovirus	*In vivo*, IT and IV	RenCa, B16F10, LLC, P815	B6D2	No significant effect	96
Feldman *et al.*	2001	Murine	Retrovirus	*In vitro* transduction	NMuLi	Nude	97%	91
Ding *et al.*	2001	Murine	Plasmid	*In vivo*, IT	Mca-4	BALB/c	51%	102
Peroulis *et al.*	2002	Murine	Plasmid	*In vitro*, stable transfectant	C6 Glioma	Wistar Rat	71%	89
Pawliuk *et al.*	2002	Murine	Retrovirus	Bone marrow transduction	T241	C57BL/6	None	92
Eisterer *et al.*	2002	Murine	Retrovirus	Bone marrow transduction	Human ALL	SCID	None	93
Shi *et al.*	2002	Human	Adeno-associated virus	*In vivo*, intramuscular	Human colon HT29	Nude	55%	100
Cichoń *et al.*	2002	Murine	Plasmid	*In vivo*, intramuscular electrotransfer	B16F10, RenCa	C57BL/6, BALB/c	84%, 60%	107

IT: intra-tumoral; IV intra-venous; IP: intra-peritoneal; ALL: acute lymphoblastic leukemia

into the tail vein of animals, high systemic secretions of endostatin in the liver and other organs were constantly observed [59,95-99]. Nevertheless, results of tumor growth inhibition ranged from 78% to none (Table **2**). A recent study made use of an adeno-associated viral vector to obtain intramuscular expression and sustain serum release of human endostatin, which resulted in partial growth inhibition of a human colon cancer xenograft [100].

In-vivo gene therapy with endostatin was further studied using direct intratumoral injections of retroviral vectors and plasmid expression vectors [88,101,102]. Surprisingly, plasmid expression vectors, supposedly the simplest gene therapy agents, were among the ones that yielded the best results ever published [88].

Other gene therapy approaches were also used, such as the *in-vivo* implantation of sodium alginate encapsulated cells genetically engineered to express endostatin [103,104], and the intramuscular or intraperitoneal administration of a plasmid expression vector [105-107]. Those studies showed partial efficacy on different preclinical models (Table **2**).

Our team designed an alternative way of delivering endostatin in preclinical models. We set out to obtain continuous intravascular release of endostatin by retrovirus-mediated gene transfer of a secretable form of murine endostatin into hematopoietic stem cells (HSC) followed by engraftment of syngeneic mouse recipients. cDNA's encoding a secretable form of endostatin was introduced into an Murine Stem Cell Virus based retroviral vector upstream of an internal ribosomal entry site/green fluorescence protein gene cassette, allowing for the preselection of retrovirally transduced bone marrow cells [108,109]. After long term bone marrow reconstitution with transduced cells, sustained, high levels of serum endostatin were obtained in the recipient mice [92]. Extensive quality controls were conducted on the secreted protein. Its authenticity was attested by micro-sequencing and its *in-vitro* activity was confirmed on BCE and HUVEC cells [92,93]. Nevertheless, we did not observe any growth retardation after subcutaneous implantation (primary tumor model) or intravascular injection (pulmonary metastasis model) of syngeneic T241 sarcoma [92]. A similar approach was used on a model of human B-lineage acute lymphoblastic leukemia (B-ALL) xenografted to SCID mice [93]. Sublethally irradiated recipient mice were transplanted simultaneously with transduced murine HSC and primary human ALL cells. Again, despite high levels of circulating endostatin, no antitumor effect could be observed [93].

2.3.3. Preclinical Pharmacokinetics

All pharmacokinetics studies of recombinant endostatin in preclinical models made use of the same commercially available ELISA detection kit (Accucyte® Kit; Cytimmune sciences inc., College Park, Maryland, USA), except for the study published by P. Blezinger in which a home-grown assay was used [105]. Baseline serum levels of endogenous endostatin, as estimated with the Accucyte® kit, ranged from undetectable to 350 ng/ml, depending possibly on the study, the mouse species, and whether controls were tumor-bearing animals or not [47,59,96,107,110].

Few studies have investigated endostatin pharmacokinetics after parenteral administration of the recombinant protein. Our group assessed serum endostatin levels following a single subcutaneous injection or 12 daily subcutaneous injections of 20 mg/kg of mouse recombinant precipitated endostatin. In this setting, we could not detect any rise over baseline serum concentrations (80-120 ng/ml) [47,92].

By contrast, high levels of serum endostatin were detected after parenteral administration of human recombinant soluble endostatin. Single subcutaneous injection of 1.5 mg/kg and 50 mg/kg transiently raised the serum levels to 161 and 4582 ng/ml, respectively [83]. Systemic concentrations of 200-300 ng/ml were maintained when endostatin was delivered continuously at 12 mg/kg/day via an implanted osmotic pump [84].

More data were published following different gene therapy approaches. The highest serum levels of recombinant proteins were achieved by means of a recombinant adenoviral vector carrying endostatin cDNA injected intravenously. Transient serum concentrations over 1,000 ng/ml were repeatedly obtained, with a maximum of 20,000 ng/ml in the experiment reported by Kuo and colleagues [59,95-99]. On the contrary, the single study using a rAAV vector showed low but sustained serum concentration of only 40 ng/ml [100].

Three studies have reported data following *in-vivo* transfection with plasmid. Using *in-vivo* intramuscular transduction, Blezinger and colleagues raised endostatin concentration to 8 ng/ml (in this experiment, control mice had endostatin serum levels below detection limits) [105]. By contrast, high levels of circulating endostatin where reported after intramuscular electrotransfer of a plasmid (600 ng/ml vs. 300 ng/ml for the control mice) [107]. Using intravenous injection of liposome:plasmid expression vector complexes, Chen and colleagues reported serum endostatin levels at 10 ng/ml and 33 ng/ml over a baseline concentration of 12 ng/ml, on days 1 and 2, respectively [101,110].

Finally, no rise in serum endostatin levels was detected after the implantation of a murine liver cell line transduced *in vitro* with a retroviral vector containing an endostatin cDNA [91]. On the other hand, our team has shown that sustained, high levels of serum endostatin were obtained after retrovirus-mediated gene transfer of a secretable form of murine endostatin into hematopoietic stem cells (up to 750 ng/ml vs.110 ng/ml for the control) [92,93].

DISCUSSION

When one considers preclinical data published on endostatin, differences in antitumoral effects from one study to another are manifest. Those differences are patent in studies with similar experimental design, but they are even more evident in studies testing different preclinical approaches (Tables **1** and **2**). Recombinant precipitated murine endostatin proteins, although almost identical in terms of sequence, preparation, storage and administration, had effects ranging from total regression of established

tumors to non-significant tumor growth retardation [47,72,77]. The same was true for soluble murine endostatin obtained from yeast [55,79] and for gene therapy procedures [48,59,96,97].

With regard to a putative dose-response effect, three studies, one with precipitated endostatin and the others with human soluble endostatin, reported discriminating dose-response curves from 0.25 mg/kg/day to 50 mg/kg/day [26,83,84]. On the other hand, endostatin from 293 cells was shown to have a stronger activity when used at 10 µg/kg/day than at 250 µg/kg/day, which, in any case, represents doses 100 to 1,000 time lower that their yeast counterpart [57]. Human soluble endostatin was shown to be slightly less active in mice than murine endostatin, thereof requiring higher doses for comparable effects [84]. Nevertheless, continuous human endostatin infusion allowing for a steady state serum concentration of 300 ng/ml had strong antitumor effects, while 3 to 10-fold higher circulating levels of murine endostatin (following gene therapy procedure) had almost no efficacy on the same tumor models [84,92,96,97]. Furthermore, if one considers gene therapy studies, it appears that experiments that yield the best results in terms of gene transfer and endostatin serum levels, also report the most disappointing data in terms of tumor control [92,96,97,100,101,105].

Additional discrepancies have been published with regard to endostatin mutational analysis. The original team first made use of a N-Terminal His-tagged protein and reported that a C-terminal-tagged endostatin produced in the same condition had no effect, either *in vitro* or *in vivo* [26]. Nevertheless, C-terminal tagged endostatin was shown to be active in other studies [57,87]. Similarly, putative mutations of the zinc-binding site were detrimental in one study but had no effect on others [57,60,83].

How could those differences be explained? Several hypotheses can be raised, but no theory can entirely explain the differences observed in the preclinical data published to date.

First, the best results published so far, actually the only report of significant and reproducible tumor regression were obtained with recombinant precipitated endostatin produced in *E. coli* [26,72]. For those experiments, it was assumed that endostatin gradually resorbed and refolded *in vivo*, but no experimental arguments support this hypothesis. Moreover, we were unable to show any rise in serum endostatin levels following such procedure [47], even though parenteral administration of an equivalent amount of soluble endostatin is responsible for high serum concentrations of the recombinant protein [83]. One may hypothesize that *in-vivo* biological modifications of the unfolded protein and/or contamination are partly responsible for the reported antitumor activity. Those unknown modifications might account for the fact that some endostatin mutants show different activities when used as precipitated or soluble forms, or in gene therapy models [57,60,87].

Secondly, endostatin activity might depend on the tumor model and the timing of endostatin administration. Several lines of evidence favor that different solid tumors have biologically different vessel endothelium [111,112], with tumor cells possibly implied along with endothelial cells in some cases [113]. Moreover, angiogenesis is a dynamic process, involving different phases from sprouting, to loop formation, branching, and stabilization [114]. Accordingly, the molecular regulation of those events evolves from one stage to the other [39]. Obviously, endostatin may be more active in a given biological context, as shown by some *in-vitro* experiments [52,57]. This could explain the variable activity of a given endostatin preparation from one tumor model to another [55,79], and also its variability within the same model, depending on the size of the tumor at initiation of treatment [80].

Third, one might assume that the total amount of protein detected by the ELISA kit used in those studies corresponds to multiple forms of truncated collagen XVIII, only part of which being biologically active. It may be that a small rise in serum levels of active protein is not detectable against the background, although sufficient to allow for the growth retardation of a transplanted tumor [47,105,110]. Moreover, the lack of anti-tumoral activity reported in the gene therapy studies which allowed for very high levels of serum endostatin, suggest that the anti-angiogenic activity of endostatin is not directly dependent on its serum level and that its dose-activity relationship might follow a U-shaped curve, as has been reported for TGF-beta and for interferon [115].

Finally, specific interaction between the tumor and its environment, particularly the immune system, might be responsible for some of the results observed in those preclinical models [116].

CONCLUSION

Despite extensive preclinical development, many questions remain to be answered with regard to endostatin mechanism of action and anti-tumoral properties. The preclinical work published to date has brought up a lot of unanswered questions, which need to be elucidated. At the moment, the best schedule of administration, clinical situation and *in-vivo* assay to monitor endostatin efficacy are not known [117].

Better knowledge of endostatin properties, both at the cellular and the molecular levels is a prerequisite before any sound progress can be made with this promising molecule. Only complete understanding of its activity will allow for the development of efficient treatment making use of endostatin at its best, either alone or in combination with other therapeutic modalities.

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ABBREVIATIONS

VEGF	=	Vascular endothelial growth factor
bFGF	=	Basic fibroblast growth factor
MMP	=	Matrix metalloproteinases
ECM	=	Extra-cellular matrix
CAM assay	=	Chorioallantoic membrane assay
BCE	=	Bovine capillary endothelial cells
HUVEC	=	Human umbilical vein endothelial cells
LLC	=	Lewis lung carcinoma
HSC	=	Hematopoietic stem cells

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